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Peptidoglycan in *Orientia tsutsugamushi* and other Rickettsiales species

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31st May 2019

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Abstract

Orientia tsutsugamushi is an obligate intracellular bacterium which causes the neglected human disease scrub typhus. The cell biology of *Orientia* is relatively poorly studied compared to other human pathogens of equivalent severity and prevalence. Due to the lack of tools for genetic manipulation, this project initially focused on developing a toolkit of probes for labelling bacteria for fluorescence microscopy. By successfully labelling *Orientia* with antibodies, dyes and chemical probes, key biological questions can be investigated.

One of these key questions is whether *Orientia* synthesises peptidoglycan. Bacterial cell walls typically contain a thick polymer structure of sugars cross-linked by amino acid bridges called peptidoglycan, which is the main determinant of cell shape and which is essential in protecting bacteria from osmotic stress. The fact that it is both essential and unique to bacteria means that eukaryotic cells have evolved to recognize it as a highly immunostimulatory PAMP, and it is also an important target for antibiotic therapy. Obligate intracellular bacteria have evolved to replicate exclusively inside host cells, and the resulting proximity to peptidoglycan receptors such as NOD1/NOD2 may cause selective pressure to reduce the amount of immunostimulatory peptidoglycan in their cell wall.

Of the two major groups of obligate intracellular bacteria, the Chlamydiales and Rickettsiales, the peptidoglycan structure in the Chlamydiales has been more extensively studied. It was previously reported that *Orientia* does not contain a peptidoglycan structure in its cell wall, even though it encodes most of the genes required for production. The research described in this thesis demonstrates that *Orientia* does possess a peptidoglycan-like structure in its cell wall. *Orientia* also has an outer membrane comprising of a network of disulphide crosslinked proteins, and this works together with the peptidoglycan-like structure to confer structural rigidity and osmotic

protection. The minimal peptidoglycan-like structure of *Orientia* presents striking similarities with that of the unrelated Chlamydiales group, suggesting convergent evolution to selective pressures of the obligate intracellular lifestyle.

Based on the similarities between *Orientia* and *Chlamydia*, a bioinformatics analysis of the peptidoglycan biosynthesis pathway was performed on all major obligate intracellular bacteria, including several Rickettsiales species. This led to the prediction that some – but not all – Rickettsiales would possess minimal peptidoglycan-like structures with similarities to those seen in *Orientia* and *Chlamydia*. This motivated the third part of this thesis, in which peptidoglycan was studied in five diverse species from the order Rickettsiales (*Rickettsia canadensis*, *Wolbachia*, *Anaplasma marginale*, *Anaplasma phagocytophilum*, *Ehrlichia chaffeensis*). This comparative study revealed differences in peptidoglycan-like structures in these organisms that were supported by bioinformatic predictions, leading to hypotheses about how differences in their life cycles and cell tropism led to various selective pressures on their cell wall composition.

Objectives

1. To develop tools to label *Orientia* for fixed and live imaging
 - To (commercially) generate and test antibodies specific for membrane proteins of *Orientia*
 - To develop a toolkit of commercially available chemical probes that could be used to label genetically intractable obligate intracellular bacteria for fluorescence microscopy imaging
2. To test whether *Orientia* possesses peptidoglycan in its cell wall
 - To investigate if *Orientia* is sensitive to peptidoglycan targeting drugs
 - To use D-alanine based peptidoglycan-specific chemical probes to determine the presence or absence of a peptidoglycan structure in *O. tsutsugamushi*
 - To measure the sensitivity of *O. tsutsugamushi* to osmotic stress
3. To identify peptidoglycan in Rickettsiales species and classify species in specific groups
 - To develop protocols for growing multiple Rickettsiales species in different cell lines (*Rickettsia canadensis*, *Anaplasma marginale*, *Anaplasma phagocytophilum*, *Ehrlichia chaffeensis*, *Wolbachia*)
 - To investigate if Rickettsiales species are sensitive to peptidoglycan targeting drugs
 - To use D-alanine based peptidoglycan-specific chemical probes to determine the presence or absence of a peptidoglycan structure in Rickettsiales species
 - To measure the sensitivity of the Rickettsiales species to osmotic stress

Scientific Contributions

In this thesis, some experiments were carried out by collaborators.

Suparat Giengham (Mahidol Oxford Research Unit, Bangkok and Public Health Research Institute, New Jersey)

- qPCR of *Orientia* labelled with CFSE, SYTO9, HADA, vybrant dyes, (**Figure 11, Figure 13, Figure 14, Figure 16, Figure 21**)
- qRT-PCR of gene expression in *Orientia* (**Figure 21**)
- qPCR of drug sensitivity in *Orientia* (**Figure 22**)
- Western blot analysis of *Orientia* with and without β -mercaptoethanol (BME) (**Figure 27**)

Jeanne Salje (Mahidol Oxford Research Unit, Bangkok and Public Health Research Institute, New Jersey)

- Protein alignment of PBP2 and PBP3 in *Orientia* (**Figure 23**)
- Bioinformatics analysis of Rickettsiales species [1] (**Figure 28**)

Janatana Wongsantichon (Mahidol Oxford Research Unit, Bangkok)

- Specificity of antibodies against *Orientia* by western blot analysis (**Figure 7**)

Peter Schumann (Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) and Suwittra Chaemchuen (formerly of Mahidol Oxford Research Unit, Bangkok)

- Meso-DAP identification by mass spectrometry (**Figure 20**)

Chapter 1

Literature review: Introduction to *Orientia* and other Rickettsiales species

1.1 Introduction

Rickettsiales are an order of alphaproteobacteria which comprise of a group of obligate intracellular bacteria and these commonly infect eukaryotes (**Figure 1**) [2]. There are three known families: *Anaplasmataceae*, *Holosporaceae*, and *Rickettsiaceae* [1]. Many species in these families cause well known zoonotic diseases and extensive agricultural damage. With better diagnostics and surveillance mechanisms, the incidence of disease is now easier to record and recent data shows that these bacteria are becoming a public health threat [3].

This thesis will focus on (i) developing tools for labelling and (ii) studying peptidoglycan in the cell wall of two main families: *Anaplasmataceae* and *Rickettsiaceae*, and especially the rickettsial bacterium *Orientia tsutsugamushi* (**Figure 2**). The presence and structure of peptidoglycan is well researched in free living bacteria. However, for obligate intracellular bacteria such as the Rickettsiales order this is not the case [1]. These obligate intracellular bacterial species are difficult to work with, as host cell culture is necessary. Also, some species such as *Orientia tsutsugamushi* and most *Rickettsia* species require biosafety containment laboratories which are expensive and difficult to maintain [4].

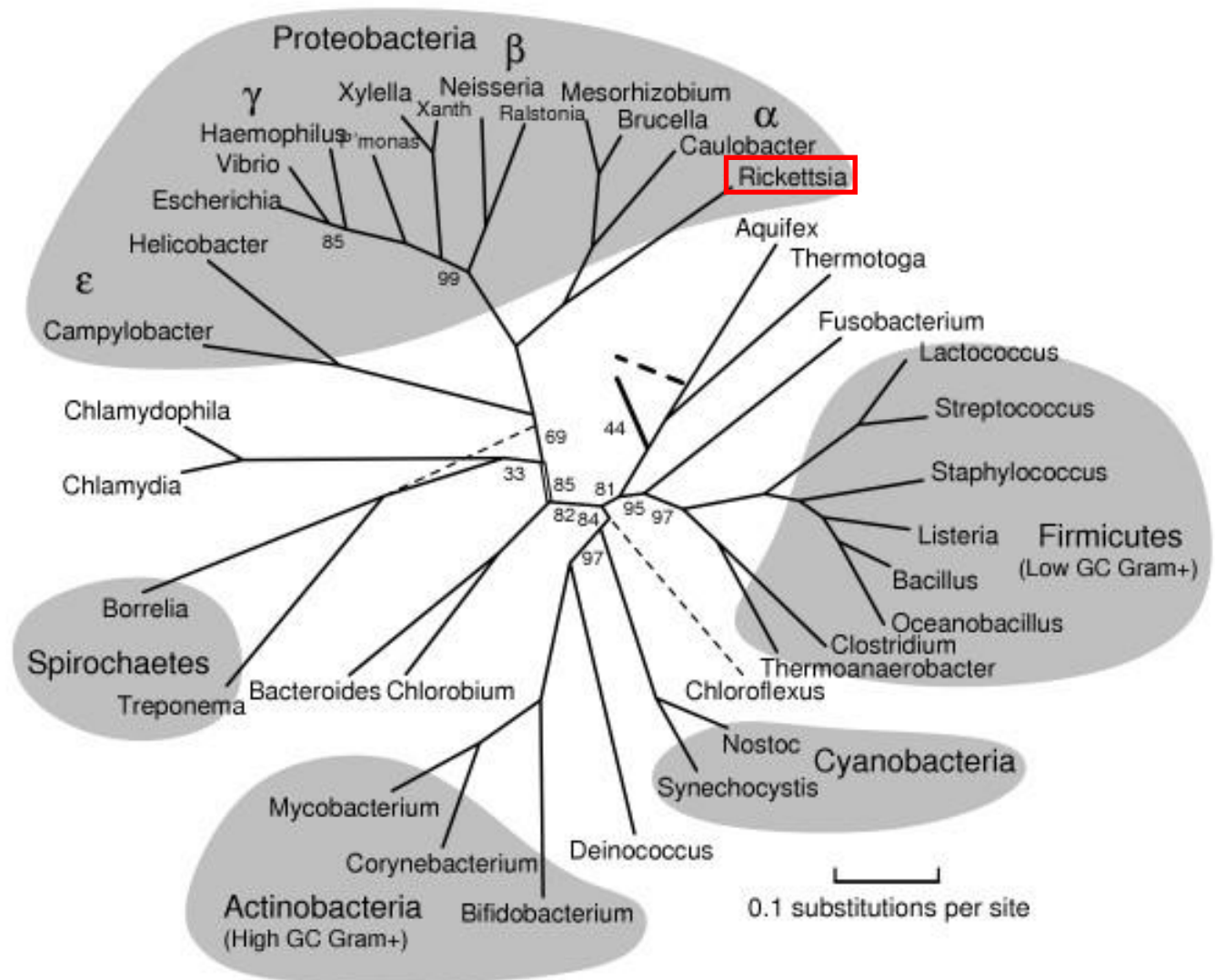


Figure 1. Phylogenetic tree of bacterial species [1]

Highlighted genus of Rickettsia. In this area of the tree, the Rickettsiales order is found.

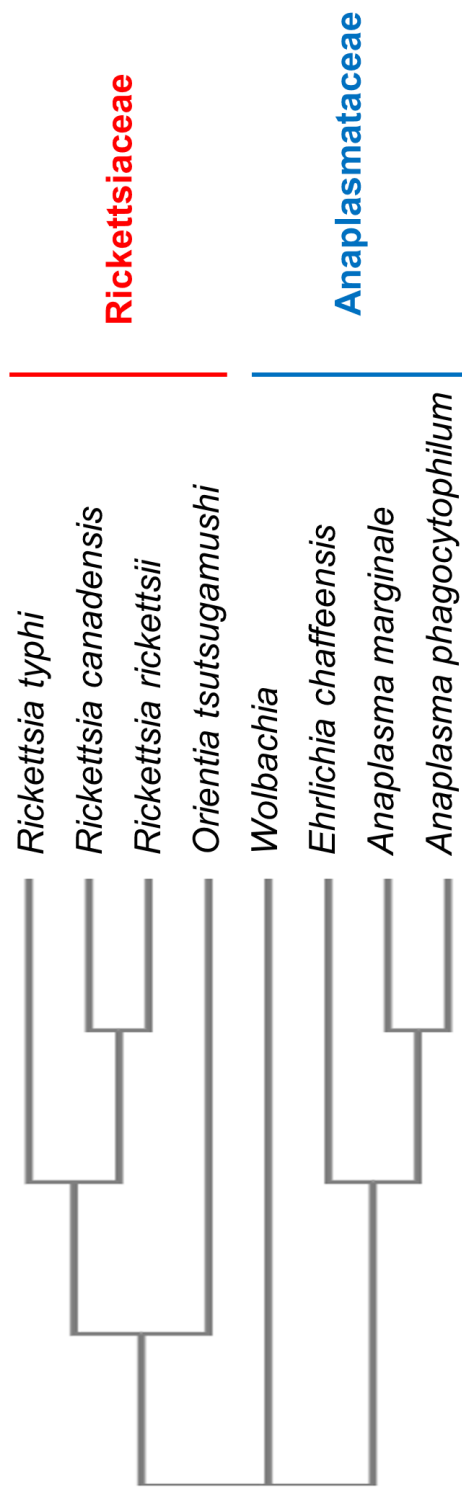


Figure 2. Phylogenetic tree of order Rickettsiales species, inferred from FtsZ gene sequences

Phylogenetic tree composed using Clustal Omega (EMBL-EBI) of the species researched in this thesis. Other Rickettsial species *R. typhi* which causes murine typhus and *R. rickettsii* which causes rocky mountain spotted fever are included in this tree for completion although they are not used experimentally in this work.

1.2 *Orientia tsutsugamushi*

1.2.a Scrub typhus

Scrub typhus is a mite-borne zoonotic disease. The word typhus originates from the Greek word 'hazy' which relates to the patients' state of mind once infected by *Orientia tsutsugamushi*. A Rickettsial Gram negative obligate intracellular coccobacillus bacterium, it is transmitted to humans and small mammals from the bite of arthropod trombiculid larval mites of the genus *Leptotrombidium*, also known as chiggers [5]. Scrub refers to the type of environment the disease was classically acquired. After hatching and in order to develop further the larval chiggers need to feed on a mammalian host, and in the process, they transfer bacteria from their salivary glands to the host antigen presenting cells (APCs). *Orientia* is able to infect a wide variety of cells in the mammalian host including professional phagocytic cells such as epithelial, macrophage, dendritic, polymorphonuclear APC and lymphocyte cells as well as non-professional phagocytic cells such as epithelial, endothelial and fibroblast cells [6].

After an incubation period of 6-21 days, various clinical manifestations may arise such as rash, eschar and fever as well as organ failure once the infection progresses. The eschar is the classic sign of the disease; this occurs where the chigger has bitten the skin and here the bacteria level will be very high, causing a high immune response and leaving the skin red and swollen [7]. Serious central nervous system complications may arise such as meningitis, encephalitis and seizures [8, 9].

The mortality associated with scrub typhus may be up to 33-45% in patients who have not been treated in endemic areas which are usually rural, but this does not consider endemic areas with immunity [10]. Risk of mortality depends on the infecting strain, the physical wellbeing of the patient, and treatment given [11]. An estimate of 1 billion people in the Asia-Pacific area are at risk of being infected with scrub typhus and 1 million new cases will develop every year [12]. This

is likely to be much higher as many cases will go undiagnosed and unreported, especially in rural areas of infection where other febrile illnesses may be more commonly known [13]. Scrub typhus is also one of the leading causes of sepsis in Southeast Asia [14].

However, cases of scrub typhus have emerged from Dubai [15] and Chile [16] indicating that the disease burden may be not just be limited to the so called ‘tsutsugamushi triangle’ area of infection.

Orientia may be treated with antibiotics such as doxycycline and chloramphenicol which inhibit protein synthesis but because of the many antigenically distinct serotypes, relapses and reinfections are common [17]. Also, treatment with these antibiotics may be dangerous in pregnant women and children. Alternative antibiotic therapy is also established, such as treatment with macrolides, quinolones and rifampicin [18].

Selection for antibiotic resistance is not a major concern, because the bacteria cannot not be released back to the natural chigger host from the human host, so there is no selective pressure for the bacteria to evolve against antibiotics. There have been strains with reduced antibiotic sensitivity reported [19] but the existence of widespread doxycycline or chloramphenicol resistance in *Orientia* has not been found [20].

1.2.b Natural ecology of scrub typhus

Orientia is found in the larval stage of *Leptotrombidium* mites which act as the vector and the reservoir of the bacteria. The lifecycle of mites in the family Trombiculidae begins with the egg which develops into the 6-legged prelarva, larva and then 8 legged protonymph, deutonymph, tritonymph, and adult. The larval, chigger, stage is the only parasitic stage. Using hair follicles to attach to mammalian skin, the chiggers form a stylostome which project into the host’s dermis for

food intake which facilitates horizontal transmission of *Orientia*. Feeding time may be from 2-7 days [18]. The infected human is a dead-end host as the bacteria cannot reinject future chiggers which may attach again but infected rodents can reinfect chiggers which attach to them.

Orientia persists in the mite line. Transovarial transmission occurs, as the bacteria is passed from the female to the eggs. Transstadial transmission occurs through the nymph and adult stages.

Although Trombiculid chiggers are the known vectors (and reservoir) in endemic regions, there may be unknown vectors which transmit *Orientia* in other parts of the world, especially the western hemisphere [21].

1.2.c Genome

Orientia was originally assigned to the genus *Rickettsia* and was named *Rickettsia tsutsugamushi*. In 1995, it became reclassified to its own genus due to phenotypic and genotypic differences such as its seeming lack of peptidoglycan and LPS, traits which *Anaplasma* and *Ehrlichia* species share [22] [23]. There are two species classified in the *Orientia* genus: *Orientia tsutsugamushi* and *Orientia chuto*. There are many different strains of *Orientia tsutsugamushi* which are genotypically and antigenically distinct. *Orientia chuto* was found in a patient who had visited the United Arab Emirates, which is far from the usual endemic area of scrub typhus infection [15]. Additionally, *O. chuto* was found in chiggers living on rodents in Kenya [24].

The first strains of *Orientia* to be fully sequenced and annotated were Boryong, isolated from a patient in Korea [25], and the Ikeda strain, isolated from a patient in Japan [26]. Six further strains have recently been completely sequenced: Karp, Kato, Gilliam, TA686, UT76 and UT176 [27].

Orientia has a circular single chromosome of roughly 2.1Mbp in size, the biggest in the Rickettsiales order [28] [27], with a core genome of 657 genes. However, between islands of core gene, the genome consists of a high number of repetitive genes which make up over 42% of the

genome. This includes the greatly amplified integrative and conjugative element (ICE), the rickettsial-amplified genetic element (RAGE) [4]. RAGE is a large genetic element which encodes over 30 genes including ankryrin genes, tra genes related to T4SS, effector protein genes, integrase and transposase genes. RAGE is found in many copies in the genome but most of these elements have been degraded by insertions and deletions, although many genes still remain functional. Similarities have been found between RAGE and gene clusters in *Rickettsia bellii*, an ancestral species.

It is unusual that *Orientia* encodes many ankyrin repeat containing proteins (Anks), as these are more commonly found in eukaryotes. It has been shown that *Orientia* Anks can be exported using a T1SS apparatus into the host cell [29] [30]. Most Anks have been found to localise to the nucleus, cytosol or the endoplasmic reticulum, and the roles of some in interacting with host cell proteins has been although the majority of the >20 Ank protein families remain uncharacterized [29] [31] [32].

Orientia encodes many copies of conjugative type 4 secretion system (T4SS) which mediates the transfer of DNA among bacteria as well as transporting effector proteins into the host cells [27]. *Orientia* has over 300 tra genes which express components of the T4SS [25]. The genes encoding the T4SS are well conserved between *Orientia* and *Rickettsia*, indicating their vital role in the intracellular lifecycle. It is unknown if the T4SS of *Orientia* is active nor what it exports.

With all the above genetic insertions, the genome of *Orientia* is not organised well and genes with related functions are not closely linked as expected. Between strains, the positions of genes do not correspond, highlighting the massive reshuffling which has occurred over time [27]. The presence of the degraded and non-coding genes would be maintained in a narrow bottleneck population like the intracellular population of *Orientia*, as selection against these genes becomes less effective [27].

These adjustments and rearrangements of the genome may be the reason why *Orientia* serotypes are so antigenically diverse, a feature which makes vaccine development nearly impossible [25].

1.2.d Requirement for host nutrients

What does *Orientia* need from the host? Firstly, pyruvate is not synthesised by the typical glycolysis pathway, but it is most likely taken up from the host instead or synthesised by an alternative pathway in which malate is needed from the host. *Orientia* does not possess acetyl CoA for the citrate acid cycle which is also imported from the host. Even though *Orientia* has most of the genes needed for oxidative phosphorylation, ATP is also acquired from the host through the ATP/ADP translocase. This allows the bacteria to use up the pool of host ATP first, before using their own resources. The ATP/ADP translocases may also import purines needed for nucleotide metabolism [33].

Orientia cannot produce amino acids so it most likely upregulates transporters which yield amino acids from the host cell instead [17]. Importantly, alanine racemase which converts L-alanine to D-alanine, vital for the structure of peptidoglycan, is absent. Alternatively, *Orientia* may take D-amino acids from the host cell or just retain L variants in the polypeptide chain of peptidoglycan [34]. Almost all genes required for peptidoglycan synthesis exist; this will be discussed later. Like other Rickettsiales, *Orientia* has the genes to synthesise fatty acids. With no LPS, the bacteria do not have the genes for the biosynthesis of lipid A, unlike *Rickettsia* species [33].

Bacteria can sense and respond to their environment through two-component regulatory systems which leads to alteration of gene expression. *Orientia* has a histidine kinase sensor system associated to proteins which are involved in managing with osmotic or oxidative stress [26]. It is currently not possible to manipulate the genome of *Orientia* and therefore it is not possible to specifically delete genes and investigate their role in the bacterial life cycle. Previous work on

gene expression analysis showed that genes responsible for protein translation were down regulated in macrophages compared to fibroblasts cells, leading to the slower growth of *Orientia* in macrophages [7]. The difference in environment in these host cells may have stimulated different responses in *Orientia*, hence the difference in gene expression.

It is still not known how *Orientia* is able to control its genes to down-regulate translation. The presence of SpoT genes may play a role in this, as these genes hydrolyse alarmones by detecting nutrient stress and responding by limiting hydrolase activity [35]. This stringent response enables bacteria to control their gene expression by regulating transcriptional repression of genes needed for the translation machinery, and upregulating genes for metabolic enzymes e.g. amino acid biosynthesis. *Orientia* is the only bacterium in Rickettsiales to encode a full-length SpoT/RelA homologue [3].

1.2.e Surface proteins

Bacterial entry into nonphagocytic host cells is mediated by the interactions between surface components on the bacteria and receptors on the host cell. At least 5 major surface antigens of *Orientia* have been identified from patient sera by western blot analysis but for most their functions remain largely unidentified. They may be involved in various functions such as bacterial entry and they are immunostimulatory. These proteins are TSA56, TSA47, TSA22, TSA110 and ScaC. The first three are the type specific antigens which are the most common surface membrane proteins [13].

The TSA56 protein is the most abundant surface protein and the most antigenically varied. Identified by the immunoprecipitation of surface proteins from highly reactive rabbit sera [36], the TSA56 is strain specific and found on the bacterial outer membrane. Its primary function is the adhesion of the bacteria to the host cell, as well as being implicated in the virulence of the bacteria.

The TSA47 protein, which is highly conserved across the strains, is also implicated in bacterial adhesion to the host cell but to a much lesser degree and may work mutually with TSA56 to initiate bacterial entry.

There are four variable regions in the TSA56 protein which are crucial in distinguishing strains [37]. The four main regions of hypervariability are all in the hydrophilic residues of the protein that are predicted to reside on the surface of the cell. It also produces the strongest immune response in patient's sera so this antigen would prove useful for a vaccine target or diagnostic marker [38].

Rickettsial bacteria encode multiple autotransporter domain family proteins and these are used to distinguish species. Each rickettsial species may have a different complement set of Sca proteins. *Orientia* encode 5 of these genes also known as the Sca (surface cell antigen) family, which are known to be involved in adhesion and immunity. These are ScaA-ScaE [12]. Although entry is predominantly initiated by the TSA56 protein, the ScaC surface protein of *Orientia* also plays an essential role in virulence, by facilitating attachment of the bacteria to the host receptor. It has been shown that ScaC in Boryong and Ikeda strains, does induce specific antibody responses and is expressed in the host cell but it only mediates bacterial adherence to the host fibronectin, therefore it is not involved in bacterial ingress [39].

1.2.f Infection and disease

Once a human is bitten by an infected chigger mite, *Orientia* multiplies at the site of infection, which is not always visible but when it becomes so is known as an eschar [40]. The primary infection site for *Orientia* were thought to be endothelial cells but it is now known that *Orientia* is also found in monocytes and dendritic cells [6]. Symptoms typically begin 7-14 days post infection and include headache, fever, rash, myalgia and lymphadenopathy.

Once infected, a proinflammatory response is activated. This results in either clearance of the pathogen or inflammatory-related damage to the organs and tissue damage. If the pathogen is not cleared by the immune response and appropriate antibiotic therapy the bacteria spreads to multiple organs including the heart, liver, lungs, spleen, kidneys and central nervous system. This can lead to multiple organ failure and death.

1.2. g Diagnostics

Scrub typhus presents symptoms similar to many other infectious diseases (headache, fever, rash) making unambiguous diagnostics based on clinical presentation difficult. When an eschar is present at the site of the mite bite this is a good indication of scrub typhus. However, eschars are not always present and bedside diagnostic tests are required. Serological diagnostic tests include the indirect immunofluorescent antibody (IFA) assay which uses a fluorescent anti-human antibody to detect specific antibodies from patient serum bound to antigens from three different serotypes of *Orientia*. Although this technique is sensitive, antigenic variation (there are over 20 different serotypes of *Orientia*) can create false negatives and the procedure requires expensive equipment [41] [42]. Also, to achieve necessary diagnosis accuracy, it is more suitable to have two specimens especially in endemic areas. Another common diagnostic test is the agglutination Weil-Felix assay which detects antibodies against antigens of *Orientia* in patient serum [43]. Though relatively simple, cheap and easily available, it does lack sensitivity and specificity [44] and this test is rarely used.

The enzyme-linked immunosorbent assay (ELISA) is another diagnostic test which involves antibody detection of IgM and IgG. Highly sensitive, it provides an objective optical density (OD) result using an automated plate reader. This generates high throughput and the test is cheap and

easy to perform [18]. However, the cut-off levels of the OD reading in ELISA testing is not well validated so may vary from location to location [45].

Another serological test is rapid point of care tests (RDTs), which detect antibody production in response to bacterial infection [46]. These are fast and cheap but need high levels of antibody to work efficiently so during early infection it may not be the best test to use [47]. Also, serological tests may not be sensitive in endemic regions of scrub typhus as patients may produce antibodies against the bacteria for prolonged periods.

The use of serological diagnostics is key when treating patients in real time. However, genotyping and genome sequencing are also an effective way of monitoring *Orientia*. Quantitative real-time polymerase chain reaction (RT-PCR), quantifies the bacterial copy number of 16s, TSA56, TSA47 and groEL genes [48]. TSA56 exhibits high antigenic variability so it is primarily used to identify bacterial strains [18]. This technique is very sensitive but a high infection may be necessary to avoid false negatives [49] [50]. Bacteria may be grown in culture before PCR, but this is difficult as *Orientia* is grown in vitro, usually in a level-3 biosafety laboratory (if the risk assessment requires this). This is also a long process and it may take up to 4 weeks to receive a positive result [42].

Multi-locus sequencing typing (MLST) which allows strain detection after DNA sequence variation in housekeeping genes, results in better evolutionary relationships between isolates. As more of the genomes of *Orientia* become publicly available, this technique may be carried out more frequently [18]. Failure to diagnose the disease efficiently will lead to ineffective treatment with β -lactams.

1.2.h The infection cycle

1.2.h.i Entry

Bacterial invasion occurs by interactions between the bacterial surface component, host cell receptors and extracellular components.

The surface component known to bind with the host cell receptor in the case of *Orientia* is the TSA56 protein, the most abundant protein on the outer cell surface. Its role in entry was discovered after protein extracts from the bacteria were incubated with host membranes which bound to a protein of TSA56, the same size as the major outer membrane protein of *Orientia* [36]. Fibronectin, a glycoprotein of the extracellular matrix, plays a role as a host cell receptor. There has been much research which has shown that many bacteria bind to fibronectin for host entry; *Escherichia coli*, *Staphylococcus* and *Campylobacter jejuni* all bind to diverse binding sites on fibronectin [38].

Fibronectin contains many multifunctional domains such as two heparin binding regions, a gelatin binding region and a central cell binding domain which contains the arg-gly-asg (RGD) motif which is needed for it to interact with $\alpha 5\beta 1$ integrin receptors, the host's transmembrane receptors. The RGD motif was found to also be implicated in *Orientia* entry as blocking this interaction using anti- $\alpha 5\beta 1$ integrin antibodies and Arg–Gly–Asp–Ser (RGDS) peptides, infection became inhibited [38]. The interaction between the RGD motif in fibronectin and the $\beta 1$ subunit of the integrin receptor is key for invasion, specifically, the antigenic domain 3 and the adjacent c-terminal region of TSA56 is the assumed fibronectin binding domain. This was determined by fusing TSA56 with glutathione s-transferase (GST) truncated fusion proteins [38]. It is thought that fibronectin domains of bacteria are not organized until fibronectin actually binds with the bacteria and this ligand binding interaction causes the systematic transition. The ScaC surface protein also has a role in adhesion with fibronectin but the details of this remains unknown [39, 51]. Another

Rickettsia bacterium, *R. conorii*, has also been shown to be internalised into host cells by receptor-ligand interactions. This occurs by the bacteria binding to protein $\alpha 5 \beta 1$ which shares homology with integrin A domains, and which allows it to bind to fibronectin [52].

Fibronectin is not the only host cell receptor *Orientia* is able to interact with for infection; it may also bind with heparin sulfate proteoglycans (HSPGs) which are also located in the extracellular matrix and the membrane of host cells at high concentration. Purified *Orientia* were shown to bind with heparin [53] indicating that HSPGs must also be involved in bacterial uptake as well as endocytosis initiation but the exact ligand on the bacterial surface involved in the interaction is yet to be identified. It has been shown that infection level does depend on the concentration of syndecan-4 on the host cell surface, which interestingly can also interact with fibronectin. HSPGs are also implicated in other *Rickettsia* species host cell adhesion so its involvement in *Orientia* uptake may be expected [53, 54].

Once the TSA56 surface protein binds to the host cell receptor, integrin activation is triggered to begin downstream signalling within the host cell. The activation of $\alpha 5 \beta 1$ integrins will in turn activate FAK, RhoA and Src tyrosine kinases. Signalling transduction adaptor proteins talin and paxilin are recruited and become tyrosine phosphorylated leading to actin rearrangements at the cell surface to result in the internalisation of *Orientia*. It is suggested that GTPase protein Rac1 effects movements of the actin filaments, to assist bacterial entry which is reported to be performed by clathrin mediated endocytosis (CME) [55]. The signalling pathway leading to CME is not fully identified.

Orientia can increase Ca^{2+} inside non-phagocytic host cells upon infection, which is beneficial to the bacteria because many signalling pathways are controlled by Ca^{2+} such as cytoskeletal rearrangements and gene expression. The increase of Ca^{2+} was found to arise from inside the cell to aid in actin rearrangement [56]. The increase of Ca^{2+} inside host cells is thought to regulate actin assembly and disassembly and suggests that interactions with the endoplasmic reticulum is

key for its release. *Shigella* too is known to induce actin reorganisation by activating the Ca^{2+} signalling pathway for long durations at the sites where they invade [57].

1.2.h.ii Autophagy

Autophagy is an essential cellular system to clear out unwanted intracellular pathogens and initiate innate immunity. Many intracellular pathogens manipulate the autophagy machinery for their own survival. Once inside the host cell, *Orientia* is able to escape the endosome and induce autophagy. Its ability to escape autophagy occurs by an unknown mechanism [58, 59]. It has been shown that *Orientia* induces autophagosomes in dendritic cells [60].

The bacteria are now free in the cytosol, similar to other *Rickettsia* but in contrast to other many other intracellular bacteria that reside within specialized vacuoles within host cells. Other obligate intracellular bacteria such as *Listeria* and *Shigella* are also able to escape autophagy. *Listeria* phospholipase proteins have been shown to degrade the phagosome membrane. As *Orientia* has phospholipase D, this could potentially play the same role in autophagy escape [61].

1.2 h.iii Microtubules

Many obligate intracellular bacteria use the host cytoskeleton for transport within the cell as well as cell to cell spread [62]. *Rickettsia* species hijack cellular actin and are able to polymerise actin to propel themselves in the host as well as pushing through neighbouring cell membranes [63]. *Orientia* are unable to polymerise actin but instead localise with host microtubules during the infection cycle.

Orientia travel to the perinuclear region of the cell after escaping autophagy, by manipulating host cell microtubules [64]. Though other obligate bacteria such as *Chlamydia*, may use microtubules

at the early stages of infection, *Orientia* are the only bacteria to utilise the microtubules of the host in this way. As *Orientia* move to the microtubule organizing center (MTOC), movement is dependent on stable microtubules [61]. Once at the MTOC, *Orientia* begin to divide by binary fission at the perinuclear region.

It is not known yet how *Orientia* interacts with dynein, which surface protein may be responsible for this or and which dynactin complex is involved in the movement of the bacteria.

1.2.h.v Budding and exit

Once division is achieved, *Orientia* move into the cytoplasm and begin their exit from the host cell, beginning at around 4 days' post infection. *Orientia* have been shown to push out of the host cell, enclosing themselves in the plasma membrane of the host cell, leaving the host cell still intact. This technique would protect them from the extracellular environment before reinfection can occur by phagocytosis. The layer of host membrane is then thought to be disintegrated in a unknown mechanism [65]. So far, only one bacterium per bud has been seen to occur (data not yet published). This unique exit mechanism is very similar to viral budding and is not known to occur in any other bacterial species. *Chlamydia* species are similarly extruded from the host cells in a pinch-like manner, but in contrast to *Orientia*, one extrusion releases hundreds of bacteria and involves the use of host actin [66].

The exit of *Orientia* has also been shown to involve of the use of lipid rafts from the host membrane [67]. Lipid rafts have been shown to be used by multiple bacteria upon entry but not exit [68]. Both TSA56 and Htr (TSA47) proteins of *Orientia* are thought to interact with lipid rafts, to initiate the budding from the host cell.

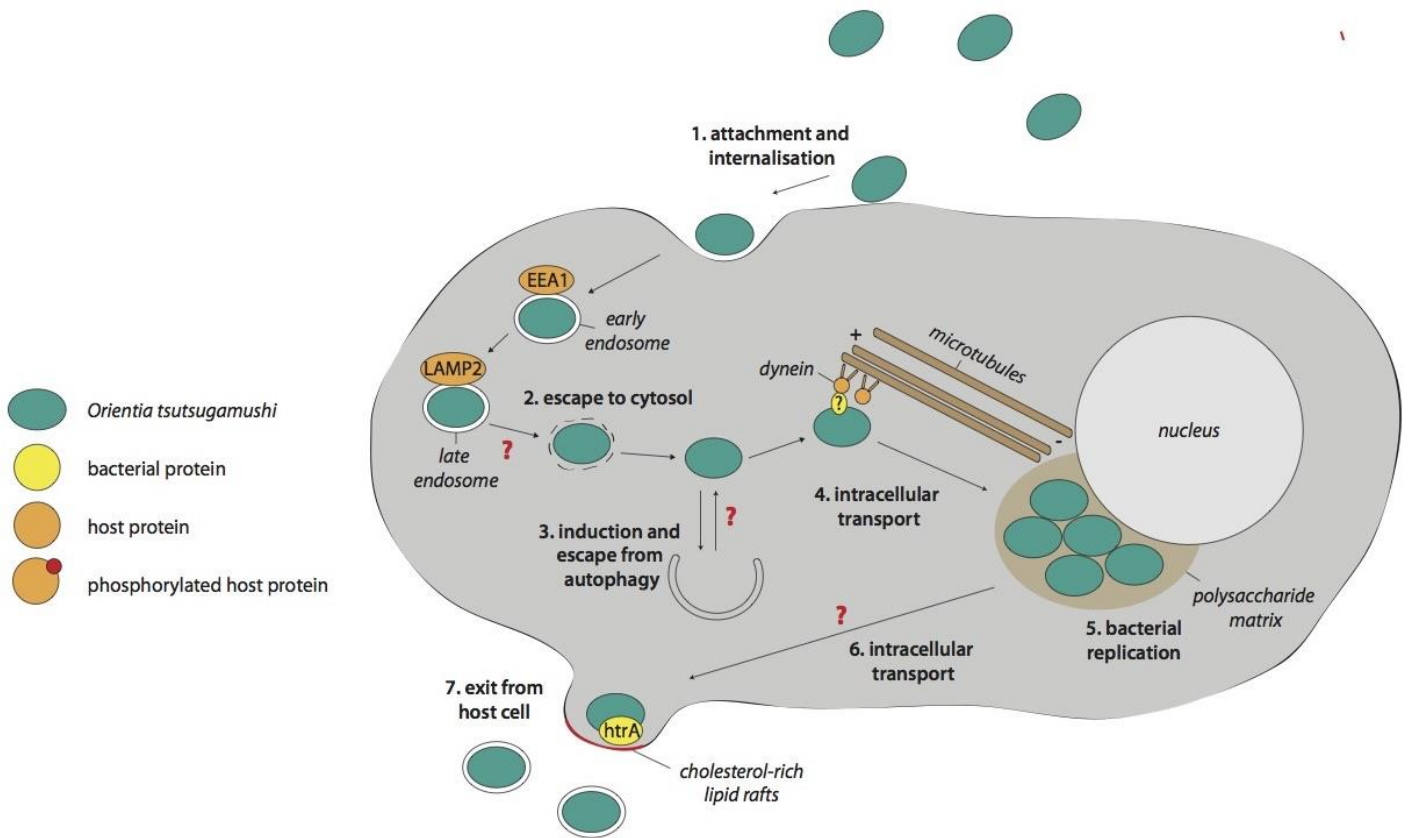


Figure 3. The *Orientia* intracellular mammalian life cycle

1. *Orientia* attach to fibronectin which activates a downstream signalling pathway in the host cell, leading to internalisation that is clathrin dependent. After entry, bacteria are enclosed in an endosome. 2. *Orientia* are then able to escape autophagy by an unknown mechanism. 4. *Orientia* utilise host microtubules to travel to the perinuclear area, aided by dynein. 5. Bacteria then undergo replication by binary fission. 6. *Orientia* then move to the host cell membrane in an unknown mechanism. 7. Once at the periphery of the host cell, *Orientia* bud out of the host, enclosed in part of the host membrane. [4]

1.3 *Rickettsia*

1.3.a. Introduction

Rickettsia bacteria were discovered by Howard Ricketts in 1906, after taking the blood of patients with Rocky Mountain Spotted Fever and infecting Guinea pigs which developed a febrile illness. He was also able to find these agents in tick cells. The discovery of the infective agent of louse-borne typhus came from Von Prowazek and da Rochalima in Europe. All later died from infection with rickettsial infections [69]. The genus consists of a diverse number of species which are associated with human disease and vectors such as ticks, mites, fleas and lice which pass the bacteria on through feeding in their saliva or feces. However, many species have also not shown any evidence of pathogenicity in humans [70].

The genus has been separated into 3 groups based on morphological, antigenic and metabolic characteristics of the species. These are: the spotted fever group, the typhus group and the ancestral group [22]. With the advent of advanced molecular tools, more *Rickettsia* species are now being discovered in a wide range of invertebrates and arthropods. *Rickettsia* species which are symbiotic to their tick hosts have been discovered and are thought to be non-pathogenic [28].

1.3.b. *Rickettsia*: Typhus group

Rickettsioses are caused by *Rickettsia* bacteria and are some of the world's oldest described diseases. The plague in Athens during the 5th century BC, was thought to have been caused by typhus caused by the *Rickettsia* bacterial species *R. prowazekii* and *R. typhi* [70].

R. prowazekii causes epidemic typhus, transmitted by *Pediculus humanus corporis* lice in colder climates. Usually transmitted in poor sanitary conditions, typhus killed over 3 million people in the last century [71]. Epidemic typhus appears repeatedly in history, especially during times of war, social upheavals and famine. Epidemic typhus has killed millions of people in history and persists today although only now in countries where public health is not well regulated. Louse-borne typhus is transmitted when human body lice excrete on human skin during feeding. The feces left behind may enter the skin as the host scratches the site of the wound or bite. Symptoms include rash, headache, fever and scratching of the skin [72].

Louse-borne diseases have re-emerged in recent times, such as in jails in Rwanda and refugee camps in Burundi. More uncommonly, homeless people in Marseilles, France were found to have seroprevalence to *R. prowazekii* [73]. Even after transmission of *R. prowazekii* occurs and no symptoms arise, years later patients can relapse. *R. prowazekii* is the only *Rickettsia* bacteria known to be able to persist in patients and this reactivation of the bacteria causes Brill-Zinsser disease [70]. *R. prowazekii* is a select agent due to its ability to be used as a biological weapon and with antibiotic resistant strains now emerging [70].

R. typhi causes murine typhus which is transmitted by fleas in warmer climates. A zoonotic disease, it is transmitted by the rat flea *Xenopsylla cheopis* to humans after exposure to flea feces through disrupted skin or via the respiratory tract. *R. typhi* occurs worldwide and case numbers are most likely under-represented due to its mildness and non-specificity in infection [74]. Symptoms include rash, fever and headache [72].

1.3.c. *Rickettsia*: Spotted fever group

The species which is most severe in this group is *R. rickettsii* which causes Rocky Mountain Spotted Fever (RMSF). It is transmitted by *Dermacentor andersoni* and *Dermacentor variabilis*

ticks in North America and *Amblyomma* species and *Rhipicephalus sanguineus* in central and south America. Common symptoms include fever with headache but there may also be gastrointestinal complications. The development of a rash is frequent, from the wrist to the trunk of the body. The classic symptom is the eschar at the site of the bite, similar to scrub typhus [69].

R. conorii causes Mediterranean spotted fever (MSF) with most cases in Europe. Transmitted by *Rhipicephalus sanguineus*, eschars can also be found in patients, but this is rare. Symptoms are similar to RMSP, with an extreme rash which can even occur on palms and soles [72].

Transmission of SFG *Rickettsia* occurs during feeding of the tick on the skin of the host. As the tick inserts its hypostome, blood is ingested and saliva (potentially containing bacteria) is injected.

1.3.d. *Rickettsia*: ancestral group

The ancestral group of the *Rickettsia* bacteria are not well known and may not be pathogenic to humans. *R. canadensis* is an ancestral species and shares characteristics with the spotted fever group (has rOmpA and rOmpB proteins) as well as the typhus group (similar G+C content and is susceptible to erythromycin). Sequence analysis of 16s show *R. canadensis* to be similar to *R. akari* or *R. felis* but places it in a distinct lineage which is not well represented. The genome is around 1.1mpb [23].

Found in *Haemaphysalis leporispalustris* ticks in Ontario, Canada in 1962 [21], from a domesticated rabbit, *R. canadensis* is transmitted transtadially and transovarially. The bacteria are able to invade the nuclei of cells and may cause febrile illness as shown by infection in Guinea pigs. *R. belli* is another *Rickettsia* ancestral species.

Species	Vector	Disease
Spotted fever group		
<i>R. rickettsii</i>	Tick: <i>Dermacentor andersoni</i> , <i>Dermacentor variabilis</i> , <i>Rhipicephalus sanguineus</i> , <i>Amblyomma cajennense</i> , <i>Amblyomma aureolatum</i>	Rocky Mountain Spotted Fever
<i>R. philipii</i>	Tick: <i>Dermacentor occidentalis</i>	Pacific coast tick fever
<i>R. peacockii</i> (east side agent)	Tick: <i>Dermacentor andersoni</i>	Non-pathogenic, endosymbiont
<i>R. montanensis</i>	Tick: <i>Dermacentor variabilis</i>	SF
<i>R. sibirica</i>	Tick: <i>Dermacentor</i> , <i>Haemaphysalis</i>	Siberian tick typhus/ Lymphangitis associated Rickettsiosis
<i>R. africae</i>	Tick: <i>Amblyomma hebraeum</i>	African tick bite fever
<i>R. parkeri</i>	Tick: <i>Amblyomma triste</i>	Rickettsiosis
<i>R. conorii</i>	Tick: <i>Rhipicephalus sanguineus</i>	Mediterranean spotted fever
<i>R. slovacae</i>	Tick: <i>Dermacentor</i>	Tick borne lymphadenitis
<i>R. honei</i>	Tick: <i>Ixodes granulatus</i> , <i>Rhipicephalus</i> , <i>Amblyomma cajennense</i>	Finders Island spotted fever
<i>R. heilongjiangensis</i>	Tick: <i>Haemaphysalis concinna</i> , <i>Dermacentor sylvarum</i>	Far Eastern tick borne Rickettsiosis
<i>R. japonica</i>	Tick: <i>Dermacentor taiwanensis</i> , <i>Haemaphysalis flava</i>	Oriental/Japanese spotted fever
<i>R. massiliae</i>	Tick: <i>Haemaphysalis juxtakochi</i> , <i>Rhipicephalus sanguineus</i>	Rickettsiosis
<i>R. rhipicephali</i>	Tick: <i>Rhipicephalus sanguineus</i> , <i>Haemaphysalis juxtakochi</i>	Unknown
<i>R. aeschlimanni</i>	Tick: <i>Rhipicephalus turanicus</i>	SF
<i>R. repullii</i>	Tick	SENLAT (scalp eschar and neck lymphadenopathy after a tick bite)
<i>R. helvetica</i>	Tick: <i>Dermacentor reticulatus</i> , <i>Ixodes ricinus</i>	SF
<i>R. asiatica</i>	Tick: <i>Ixodes ovatus</i>	Unknown
<i>R. tamurae</i>	Tick: <i>Amblyomma testudinarium</i>	SF
<i>R. endosymbiont</i>	Tick: <i>Ixodes</i>	Non-pathogenic
<i>R. monacensis</i>	<i>Ixodes ricinus</i>	SF
<i>R. akari</i>	Mite: <i>Allodermomyssus sanguineus</i>	Rickettsial pox

<i>R. australis</i>	Tick: <i>Ixodes holocyclus</i> , <i>Ixodes tasmani</i>	Queensland tick typhus
<i>R. felis</i>	Flea: <i>Ctenocephalides felis</i> / <i>Aedes albopictus</i>	SF
<i>R. hoogstralii</i>	Tick: <i>Haemaphysalis sulcata</i> , <i>Carios capensis</i>	Unknown
Typhus group		
<i>R. prowazekii</i>	Lice: <i>Pediculus humanus corporis</i> Tick: <i>Amblyomma imitator</i> Flea:	Endemic typhus/Brill-Zinsser disease
<i>R. typhi</i>	Flea: <i>Xenopsylla cheopis</i> , <i>Ctenocephalides felis</i> lice/mites	Murine typhus
Ancestral group		
<i>R. canadensis</i>	Tick: <i>Haemaphysalis leporispalustris</i>	Unknown
<i>R. bellii</i>	Tick: <i>Amblyomma</i> , <i>Ixodes loricatus</i> . <i>Acanthamoeba</i>	Unknown

Table 1. Rickettsial species and the diseases caused in humans

1.3.e. Cell biology

Rickettsia are rod shaped and have a highly conserved genome, at around 1.1Mb [75]. As they live free in the cytosol of host cells, genes required for sugar metabolism as well as lipid, amino acid and nucleic acid production are not needed [76]. *Rickettsia* species encode a glycerol phosphate transporter (glpT) which imports glycerol phosphate from the host to allow glycolysis and the citric acid cycle to occur. ATP is also imported via ATP/ADP translocases [33]. Like many Rickettsiales species, *Rickettsia* species display a T4SS with the presence of tra clusters [77].

All species have an affinity to infect endothelial cells which line the blood vessels in humans and animals. Exceptions to this include *R. conorii* which can infect a wide range of cells and the target of *R. akari* is mainly macrophage cells. Endothelial cells are known to be vital in the role of host immune responses and inflammation so by infecting these cells, *Rickettsia* bacteria are disrupting the entire vascular system [71].

Entry into host cells is by phagocytosis or by inducing phagocytosis in non-phagocytic cells [78] (**Figure 4**). A zipper-like invasion mechanism is thought to occur for most *Rickettsia* species, similar to what is known in *Listeria* and *Yersinia*, as the bacteria ligands interact with host ligands to allow host membrane to 'zipper' around the bacteria aided by actin polymerization. There have been 17 surface cell antigen (sca) proteins found in *Rickettsia* species which resemble autotransporter proteins [28]. The major surface proteins of SFG *Rickettsia* bacteria include many surface cell antigens such as rOmpA and rOmpB [79] [80]. rOmpB is the more abundant of the two and TG do not express rOmpA. Adhesion is key to infection and begins with the interaction between rOmpB and protein kinase, Ku70, found on the cholesterol rich regions of the plasma membrane as well as in the nucleus [81] [52]. On the plasma membrane, ku70 mediates cell to cell adhesion and interactions with fibronectin by forming a heterodimer with ku80 [79]. For most rickettsia species, this interaction between the rOmpB β -peptide domain and ku70 then triggers the upstream signaling pathway, via cdc42 GTPase, phosphoinositide 3-kinase and the activation

of the ARP 2/3 complex for actin polymerisation. Entry is actin dependent but may also be dependent on clathrin or caveolin 2 dependent endocytosis [71, 79]. TG *R. prowazekii* adheres to host cells using adhesion proteins Adr1 and Adr2, and may not need rOmpB for entry [82].

After entry, *Rickettsia* are able to quickly escape the lysosome which may be due to phospholipase A₂ activity, as shown for *R. typhi* (but no gene has been found to support this) [78]. The Rickettsiaceae family are unique in that they can escape the host phagocytic vacuole to reside free in the cytoplasm. This may be advantageous to them as they have direct access to host metabolites but also very risky to be exposed to components of the host cell which can trigger the host immune response.

Once free in the cytosol, rickettsia divides by binary fission. The SPG *Rickettsia* undergo actin based motility to transport themselves around the host cells, which is dependent on *Rickettsial* protein RickA [83] and occurs 24-48 hours after infection. The formation of the actin tails also allows the bacteria to protrude into neighboring host cells without being in the extracellular environment. *R. parkeri* were found to polymerise shorter tails during the first 12 hours of infection but at 24 hours, actin tails become longer and more frequent [84]. This pattern may differ according to the *Rickettsial* species and its infection cycle details. Also, rickA may interact with host actin but sca2 is thought to drive the motility of the bacteria in a ARP 2/3 dependent manner [85].

With no rickA, TG *Rickettsia* do not polymerise actin tails, with the exception of *R. typhi*, which polymerise very short tails [28]. TG bacteria lyse the host cell after division to infect neighboring host cells. This means that the bacteria number is usually very high in contrast to SFG intracellular bacteria numbers.

Rickettsia species are usually transmitted to arthropod host progeny via vertical transmission with some cases of male mortality in beetles and ladybirds [86].

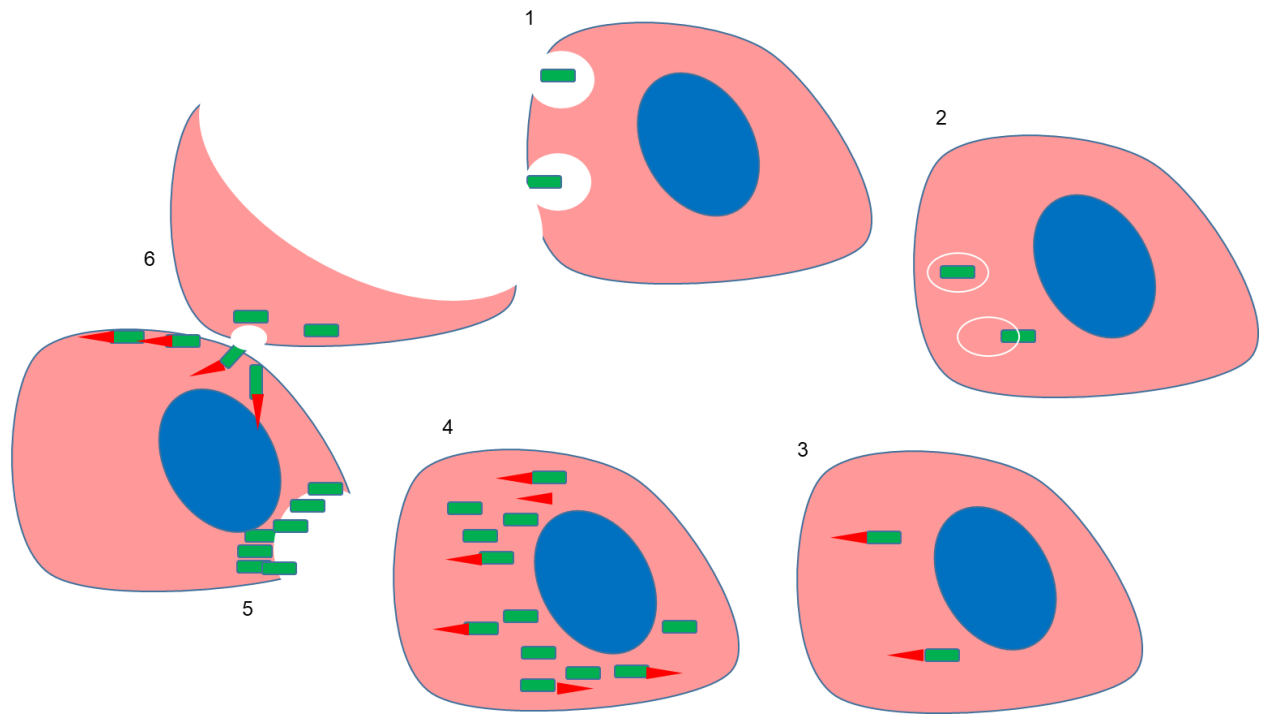


Figure 4. The *Rickettsial* intracellular mammalian life cycle.

1. *Rickettsia* attach to the host cell via adhesions rOmpA and/or rOmpB to host cell receptor Ku70 which becomes ubiquitinated. After actin rearrangement, bacteria are able to enter the cell in a zipper-like mechanism. 2. *Rickettsia* become enclosed in endosomes after entry in the cytoplasm. 3. *Rickettsia* escape the endosome as bacterial enzymes, phospholipase D and tlyC lyse the vacuole membrane. 3. Bacteria are free in the cytoplasm and may polymerise actin to move around the cell by activation of Arp2/3 by RickA. 4. Replication occurs by binary fission. 5. Typhus group *Rickettsia* will disseminate by lysing the host cell 6. Spotted fever group *Rickettsia* infect neighbouring cells by pushing through the host membrane, using actin polymerization to do so.

1.4. Anaplasmataceae

1.4.a. Introduction

There are 4 distinct genera in this family: *Anaplasma*, *Wolbachia*, *Ehrlichia* and *Neorickettsia*. Whilst all are obligate intracellular bacteria, the bacteria species in *Anaplasmataceae* all exist in membrane bound vacuoles inside the cytoplasm of host cells (**Figure 5**) which may be vertebrates or invertebrates. This contrasts with *Rickettsia* and *Orientia* species which are free in the host cytosol after entry.

These bacteria are quite different to the closely related *Rickettsia* species. *Anaplasmataceae* are sensitive to mechanical stress and osmotic changes. They are also pleomorphic with a lack of LPS and in some cases peptidoglycan (as shown in this thesis).

1.4.b. *Anaplasma marginale*

1.4.b.i. Disease

The *Anaplasma* species are transmitted by ticks, and unlike the other species in *Anaplasmataceae* they cause severe disease in livestock and humans. *Anaplasma marginale* causes bovine anaplasmosis, common in cattle and transmitted worldwide. First described in 1910 by Arnold Theiler, it causes economic losses such as death, abortion, infertility and weight loss in cattle [87]. Although it is more common in cattle, it's also found in bison, deer, buffalo and antelope. The tick populations are controlled by the use of acaricides which is now problematic as ticks become resistance to the pesticide and it can be traced in milk and meats [88]. Vaccines are used to prevent anaplasmosis which induce protective immunity but do not prevent persistent infections.

More than 20 species of ticks transmit *A. marginale* to livestock, including *Rhipicephalus* and *Dermacentor* species, but the most common is *Rhipicephalus microplus*. Animals which recover from mild anaplasmosis may be reservoirs of the bacteria for life and can re-infect ticks. The bacteria may also be passed by gestation from the cow to calf. Tick transmission may occur transstadial or intrastadial but not transovarial [89]. *A. marginale* infect erythrocytes which causes the mild to severe hemolytic disease. Infected erythrocytes are then phagocytosed by bovine reticuloendothelial cells, resulting in anemia [88].

1.4.b.ii Cell biology

A. marginale has a circular genome at 1.2-1.6 mbp [90]. The diversity of *A. marginale* can be classified based on their major surface proteins (MSP), 6 of which are well known. For instance, gene Msp1a can distinguish strains worldwide based on the tandem repeats on the N-terminal. It is used as a marker for disease severity and multiplication in cattle and ticks. MSP1a is vital for infection and transmission in ticks and is also immunostimulatory in cattle. It is the best characterized surface protein as it is known to bind with erythrocytes and tick cells. It is not yet known what MSP1s binds to on the host cells or how the bacteria enter [87]. MPS1 is also used a genetic marker to distinguish *A. marginale* strains, in particular MSP1a, MSP4 and MSP5 [91].

A. marginale can grow in vitro in various endothelial and epithelial cell lines as well as insect cell lines derived from mosquito and tick cells.

The life cycle of *A. marginale* is complicated and must coordinate with the feeding cycle of the tick. At the site of infection, *A. marginale* will develop in membrane bound vacuoles. The first form of the bacteria is the reticulated cell form which may divide by binary fission. This results in large colonies with hundreds of bacteria known as morulea. The reticulates then transform into the

dense cell form which are infective for a short period and able to survive outside of the host cells. It is the dense form that are transmitted to cattle from the ticks during feeding [92].

1.4.c. Anaplasma phagocytophilum

1.4.c.i. Disease

This species causes tick-borne fever (TBF) in animals and humans. The disease has been known for more than 200 years and transmission occurs in a broad range of hosts, from ticks to sheep, goat, cattle, horse, dog, cat, reindeer, squirrels, raccoons and human [93]. Young animals are usually affected, and symptoms range from fever, anorexia and low milk yield [94].

In humans, this febrile illness is known as human granulocytic anaplasmosis (HGA) and involves the existence of intravacuolar colonies named morulae in the cytosol of granulocytes [95]. It is an emerging infectious disease in humans and often goes unreported. Symptoms include chills, fever, malaise and myalgia. If the patient is immunocompromised, elderly or untreated, the disease can be fatal [87].

A. phagocytophilum is found in tick species Ixodes in the US, Europe and Asia [93]. As ticks take up the bacteria during feeding, bacteria are transmitted transtadially from nymph to adult during metamorphosis, and then transmitted again to human or animal. They unusually can persist in neutrophils which should naturally destroy them [94].

1.4.c.ii. Cell biology

Anaplasma phagocytophilum was once known as *Ehrlichia phagocytophilum* due to their close relation. Bacteria are around 0.4-2µm in size and are pleomorphic coccus shaped. The outer membrane is ruffled and there are no genes present encoding LPS or peptidoglycan synthesis. The lack of membrane rigidity may give it plasticity inside the inclusion bodies inside granulocytes which must travel through tight capillaries [96].

The genome size is around 1.47mbp, 12.7% of which consists of repetitive DNA [94]. They do not have plasmids or transposable elements but do have a T4SS and a partial glycolysis pathway, the ability to metabolise pyruvate and most nucleotides [97]. There is little variation between strains due to minor differences in groESL or 16S which means distinguishing strains is difficult for diagnosis. Strains may also differ in host infectivity [87].

A. phagocytophilum consist of surface membrane proteins, p44 and Msp2 which are 42-49kDa and are encoded for by the msp2 gene family [98]. These are transmembrane beta-barrel proteins which display porin activity to allow the transport of hydrophilic nutrients. Antigenic variation can be found in the loop region of the protein which is responsible for the persistence of the infection in the host. The genome of *A. phagocytophilum* also displays homologs of Type 1 and Type 4 secretions systems [99] [100].

Like *A. marginale* and *Chlamydia* species, they also display biphasic development during their intracellular life cycle; in the larger reticulate form and the smaller dense form. Only the dense form can bind with mammalian cells whereas both reticulate and dense can bind with tick cells. Once the dense form enters new host cells, they will reside in the host-derived vacuole and transform to reticulate cells within 12 hours, this is when replication occurs. After 24 hours, there will be large numbers of reticulate bacteria inside inclusion vacuoles which expands during replication, and after 36 hours, these bacteria will transition to dense form to re-infect host cells

[95] [101]. The size of the bacteria is important for uptake; as larger bacteria may be taken up via a different pathway (association with LAMP-1 proteins have been shown [102]) than smaller dense bacteria in host cells. This indicates that both reticulate and dense forms of *A. phagocytophilum* must utilise different receptors and adhesions when entering a range of host cells.

The dense form of *E. chaffeensis*, another Rickettsiales, presents outer membrane proteins which have been identified as adhesions, which contrasts with the reticulate form. The dense form of *A. phagocytophilum* is also known to interact with the host cell. This indicates that the expression of the adhesins needed for eukaryotic interactions, must occur during the differentiation stage from reticulate to dense form [101].

The entry mechanism of *A. phagocytophilum* has been studied frequently in HL-60 endothelial cells. The host cell receptor, P-selectin glycoprotein ligand-1 (PSGL-1) initiates a Syk (spleen tyrosine kinase) and ROCK-1 dependent signaling cascade which promotes the uptake of the bacteria [103]. The PSGL-1 receptor is capped with a o-glycan which has a terminal tetrasaccharide. The interaction between the bacteria and this terminal tetrasaccharide may promote cellular entry or adhesion or both. Since ROCK-1 is a regulator of actin organization, the bacteria binding to PSGL-1 results in manipulation of actin for entry. HL-60 cells are not phagocytic but have presented engulfment of bacteria via their membrane extensions [95].

The entry of *A. phagocytophilum* in tick cells is dependent on bacterial surface proteins Msp2, Asp55 and Asp62 [104]. Entry is also dependent on host lipid rafts but the signaling pathway here is not well understood. It is also known that *A. phagocytophilum* interacts with GPI-proteins and cholesterol-associated membrane proteins, flotillin 1 but not clathrin [105].

How do the bacteria persist in neutrophils, their natural niche, for so long? Usually neutrophils would kill microorganisms by withholding nutrients or fusing phagosomes with granules containing

antimicrobial peptides and lysosomal hydrolytic enzymes. But *A. phagocytophilum* can avoid the lysosome pathway and its inclusion vacuole lacks endosomal characteristic receptors and molecules. The inclusion does co-localise with various Rab proteins which regulate endocytic recycling, and takes host cholesterol for its expanding double lipid inclusion membrane [106]. *A. phagocytophilum* does not have any of the genes which encode for PAMPs peptidoglycan or LPS. Therefore, it is able to avoid being recognized by the NOD-1 receptors which are expressed on the surface of granulocytes but still activates Rip2, a downstream adaptor protein of NOD1. [107]. The bacteria are thought to down-regulate TLR1 instead. This has allowed them to remain inside the host without being immunostimulatory [97].

Bacteria can be grown in vitro in myeloid or granulocytic cells and has successfully been propagated in human promyeoblast HL-60 cells, myelomocytic THP-1, endothelial and various insect cell lines [88]. Division in HL-60 cells occurs by binary fission with division beginning by 12 hours with the induction of dense cells and formation of morulae. Bacteria load appears highest at 24 hours inside cells [95].

A. phagocytophilum, like other Rickettsiales, uses the T4SS but it is slightly different. The system may form a transmembrane channel to transfer DNA and proteins into eukaryotic cells as well as hijacking various signalling pathways [108] [109]. The T4SS may secrete virulence factors which are responsible for disrupting innate immunity and inhibiting host cell apoptosis. The bacteria may express different types of the T4SS in their tick host compared to the mammalian host, so that the vir genes are upregulated differently in both systems [110].

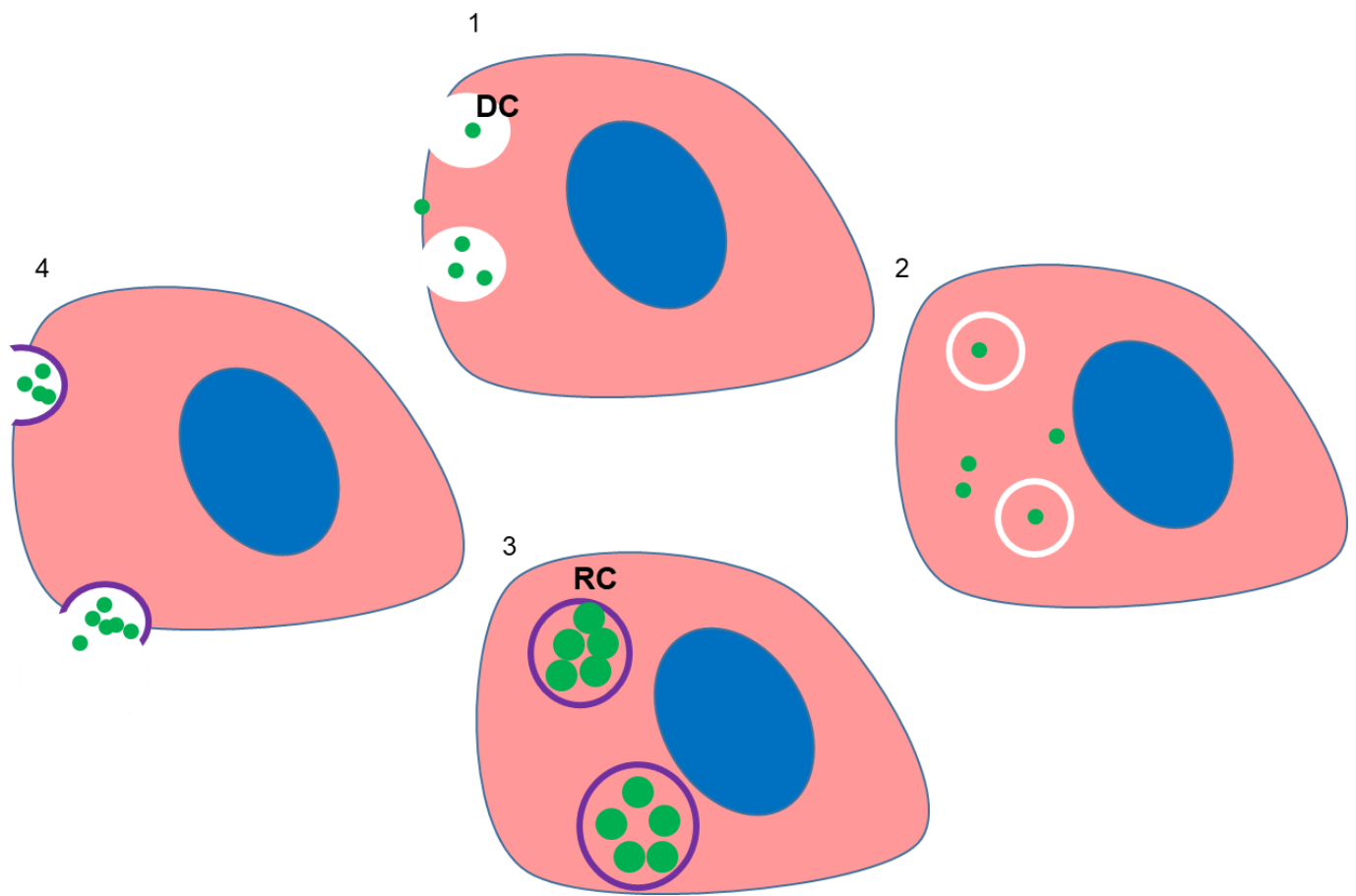


Figure 5. Anaplasmatidae intracellular mammalian life cycle.

1. Bacteria enter host cells in dense core infectious state. 2. After capture in the phagosome, no fusion occurs with the lysosome. 3. Instead the reticulate form, called the morulae develop after division. 4. Morulae then mature into the infectious dense core cells and these are released by host cell lysis or exocytosis.

1.4.d. *Ehrlichia*

1.4.d.i. Disease

The genus *Ehrlichia* includes species such as *E. chaffeensis* which causes human monocytic ehrlichiosis (HME) and *E. muris* and *E. ruminantium* which cause disease in animals such as cattle, dogs, cats, goats and rodents [111]. HME is now classified as an emerging disease by the CDC and many cases go undiagnosed or unreported with most cases emerging in elderly or immunocompromised patients [112]. *Ehrlichia* species can infect monocytes, macrophages and dendritic cells [113].

All species are transmitted by ticks and can replicate in the tick and the mammalian host. It is mainly the lone star tick *Amblyomma americanum* which transmits *Ehrlichia* species to the host [114]. These ticks are most commonly found in south central USA, especially in the summer months when ticks are more likely to bite animals or humans, but cases have also been reported in Europe, Asia, Africa and South America. White tailed deer also act as a reservoir for the bacteria [115].

Around 9 days after the tick bite, patients present headache, fever and body aches and sometimes rash. As the bacteria infection develops, the patient may experience vomiting, diarrhea, nausea and occasionally respiratory symptoms [111]. These symptoms are very similar to *Rickettsial* infections, but the mortality rate is much lower for Ehrlichiosis. Patients are usually diagnosed by PCR before given doxycycline [116]. As there is no vaccine, the prevention of tick bites is key.

1.4.d.ii. Cell biology

Ehrlichia is a pleomorphic coccus shaped bacterium with a genome size of up to 1.3Mb, coding 1200 proteins. It lacks many genes needed for metabolism and amino acid biosynthesis which most likely are taken from the host environment but does encode genes needed for aerobic respiration and nucleotide biosynthesis [33]. Its major outer membrane protein, 1/P28 is conserved amongst the *Ehrlichia* species [117] .

Other surface proteins include TRP47, TRP32, TRP120, Omp28, T4SS proteins, *Ehrlichia* surface protein 73 (Esp73) and *Ehrlichia* entry triggering protein (EtpE). Some of these surface proteins are orthologs of *A. phagocytophilum* surface proteins [115]. Bioinformative studies have shown that 18 proteins are likely to be outer membrane proteins (OMPs). These are most likely to be involved in interactions with the host cell for entry (map 1 proteins) as well as virulence factors of the T4SS [114]. This study also identified 6 new OMPs which could be vaccine targets. Many genes also showed inhibition of infection in deer when mutated which indicates which genes are important for virulence.

In vitro, *Ehrlichia* can grow in a range of cell lines from endothelial, epithelial, monocyte, macrophage and tick [118] [111].

Ehrlichia are able to enter host cells manipulating the movement of host filopodia by interfering with the cellular cytoskeleton by influencing host gene expression. This allows them to disseminate from cell to cell without entering the extracellular space and thus allowing them to avoid the host immune response [119]. *Listeria* also manipulate filopodia to exit host cells and spread to neighboring cells.

Like *Anaplasma* species, *Ehrlichia* have two morphological distinct states. The infectious form called the dense form (DC) enter host cells by adhering the invasion c-terminal region of EtpE to GPI anchored DNase X receptor, which is enriched in cholesterol in the cell membrane. These

binding permits entry as the host cytoskeleton remodels and engulfment of the bacteria is managed by filopodia. The entry of *Ehrlichia* into cells is also dependent on host cell caveolin-1 and tyrosine phosphorylated proteins [115] but not clathrin which is associated with endocytosis so entry is most likely occurs by receptor mediated endocytosis[120].

After entry, *Ehrlichia* can manipulate vesicular trafficking to avoid being delivered to lysosomes. They then differentiate in membrane bound vacuoles called the *Ehrlichia* containing vacuole (ECV), which resembles an early endosome. In the ECV, T1SS effector proteins are secreted which allows avoidance of the host immune response. Differentiation then occurs to the reticulate bodies (RB) which fuse with transferrin (TfR) endosome. This allows the bacteria to extract iron from the host cell as well as halting the JAK/STAT cell signaling pathway to avoid the innate response [121]. The RB form can then escape the lysosomal pathway by secreting T4SS effector protein ECH_0825 to prevent apoptosis and the production of reactive oxygen species (ROS) [114]. Division then occurs by binary fission to form a large colony known as the morula. Morulae increase over time and finally become loose to exit host cells, spreading cell to cell via filopodia engulfment [122]. This allows the bacteria to avoid interaction with the extracellular environment and immune responses. The use of filopodia to move to neighboring cells is actin dependent with the need for calcium and iron influx. The TRP proteins such as TRP20 mediates actin polymerization by interacting with the ARP2/3 complex [121].

Ehrlichia can prolong their survival by mediating TRP120 host interactions by misusing the SUMOylation (small ubiquitin-related modifier) pathway [114]. *Ehrlichia* may also evade host responses by interacting with mitochondria via T1SS and T4SS proteins. TRP proteins such as TRP120 and TRP47 are expressed in the dense form and are able to interact with many pathways of the host cell including entering the host cell nucleus to modify host cell signaling pathways at the protein synthesis level [114].

The bacteria taken from tick cells and infected into mice presented a longer infection than bacteria taken from vertebrate host cells [123].

1.4.e. *Wolbachia*

1.4.e.ii. Disease

Generally, *Wolbachia* is referred to just by its genus name because of confusion about whether all species belong to *W. pipentis*. The main species is *Wolbachia pipentis* and there are 8 super-groups termed A-H. These super-groups are generally associated with one type of invertebrate, A, B, E, F, G, H in arthropods and C and D in filarial nematodes [112] but some still have not been placed in a specific subgroup. Although it is possible to culture *Wolbachia* in mouse and mosquito cell lines, there is no evidence of the bacteria infecting mammals like other alpha-proteobacteria species [124].

Wolbachia species are quite different from other Rickettsiales. They do not infect vertebrates as most Rickettsiales do but more commonly infect a broad range of invertebrates, to which they share unusual relationships. Maternally transmitted, *Wolbachia* do not tend to kill their host, with the exception of a few strains, but act as a symbiont, forming a mutualistic relationship instead [125]. The bacteria are well known to be required for the survival and the fertility of the host.

It is thought that over 65% of insect species are infected with *Wolbachia*. The mutualism relationship between the bacteria and its host is most interesting. This relationship may even determine the survival of the host as studies have found tetracycline treatment in *Onchocerca ochengi* also caused host fatality [112].

Wolbachia induce interesting host manipulations which show reproductive parasitism. These include: the feminisation of male insect; the production of unfertilised eggs in females; death of male progeny (through testes infection); and cytoplasmic incompatibility (when the uninfected female egg cannot be fertilized) [126]. The latter is the most frequent and it reduces the transmission of females which are uninfected or infected with a different *Wolbachia* strain [28].

These unusual properties, especially cytoplasmic incompatibility, of the bacteria make it a suitable biological system to control vector-borne diseases. *Wolbachia* has recently been used experimentally to control dengue, a viral disease which affects 5 continents of the world and is transmitted by mosquitos of the genus *Aedes* [127]. These mosquito species are naturally not infected with *Wolbachia* but are an important vector. By infecting female mosquitos with strains wMelPop or wMel, the viral lifetime shortens drastically so that transmission is reduced. Therefore, releasing infecting female mosquitoes in urban areas can control disease. This is possible as *Wolbachia* inhibits dengue viral replication and growth [128]. *Wolbachia* may achieve this by extracting cholesterol from its host cells to allow its accumulation which negatively affects viral growth [129]. *Wolbachia* has also been shown to trigger the innate immune response of the insect to the virus. Infection of bacteria activates the immune deficiency and Toll pathway in the mosquito which also promotes the long term colonisation of the bacteria [130]. As this cements its symbiotic relationship with the host, *Wolbachia* are free to manipulate and increase the host's resistance to other pathogens [130].

Once in the insect, it is impossible to eradicate the *Wolbachia* strain and most of the time, one insect can be infected with multiple strains of the bacteria, which coexist together [128]. It has recently been shown that the addition of *Wolbachia* to mosquitoes reduces the levels of arboviruses including chikungunya and Zika, both of which are transmitted by *Aedes* mosquitoes. This is currently being tested as a control measure in clinical trials [131, 132] .

The long-term effects of *Wolbachia* additions to mosquito must be considered. Over time, dengue viruses can reduce their 'extrinsic incubation time' in the mosquito which needs to be completed before transmission if selection occurs. *Wolbachia* could also reduce the deleterious effects they exhibit on the virus [133] [128]. These are just a few examples of what could occur over time as trials take place for disease control.

1.4.e.ii. Cell biology

The genome of *Wolbachia* is highly recombinogenic as shown by the distant relationship between three of the genomes published. The genome is around 1.0-1.8Mb with high number of repeats and ankyrin repeats [126]. Its high number of repeats is only second to *Orientia* in the Rickettsiales family [134]. Diverse genomes may reflect the divergent molecules the bacteria target in various host cells, but the small genome also reflects the need for only genes needed for their parasitic lifestyle.

Like many of its relatives, *Wolbachia* has a T4SS which is likely used to transport proteins and nucleic acids in and out of the cell but also in the case of *Wolbachia*, it is used to induce the reproductive abnormalities which the bacteria inflict on the host. Also, ankyrin repeat domains have also been found in *Wolbachia* strains which infect *Drosophila melanogaster* [124]. These ankyrin proteins may also play a vital role in the mutualistic relationship between the bacterium and the host. Also unusual is the presence of the virus-like elements which may be transcribed and expressed by a lytic cycle, found in strain wMel. Furthermore, phages may be able to spread ankyrin genes within the bacteria [135].

Wolbachia have three major surface proteins: wsp and its paralogs wsp1 and wsp2 [28]. After entry inside host cells, *Wolbachia* is thought to remain inside the host cell cytoplasm, residing in a host derived vacuole. There is not much known about the activity inside the vacuole or the infection cycle which follows.

There may be a link between the bacterial growth and the host cell cycle. *Wolbachia* growth is high in early embryo development for *Aedes albopictus* but then decreases once the host cells decrease cell division [124]. As well as the host division cycle, *Wolbachia* may play a role in regulating host apoptosis. Coevolution has resulted in the host cell outsourcing the regulation of apoptosis to the bacteria instead [133].

	<i>Anaplasma marginale</i>	<i>Anaplasma phagocytophilum</i>	<i>Ehrlichia chaffeensis</i>	<i>Rickettsia</i>	<i>Orientia tsutsugamushi</i>	<i>Wolbachia</i>
VERO Epithelial	★ [88]		★ [136]	★ [137]	★ [138]	
THP1 Monocyte		★ [139]	★ [122]			
C636 A albopictus	★ [140]	★ [140]	★ [140]			★ ▼ [141]
Aa23 A albopictus	★ [142]			★ [143]		★ ▼ [144]
RF/6A Endothelial	★ [145]	★ [146]	★ [140]	▼ [147]		
PTK2 Epithelial						
DH82 Macrophage			★ [148]			
ISE6 Tick	★ [149]	★ ▼ [150]	★ [151]	★ [23]		
Trombiculid Mite					▼ [152]	
U937 Monocyte		★	★ ▼ [153]			
HL-60 Promyeloblast		★ [98]				
HMEC-1 Human Endothelial		★ [97]		[154]		
BCE C/D Cow Endothelial	★ [145]	★ [145]				
L929 Fibroblast			★ [136]	★ [155]	★ [138]	★ [156]
Erythrocytes	▼ [157]					
Leukocytes		▼ [88]				
Neutrophils		▼ [97]	▼ [158]			
Eosinophils		▼ [97]				

Table 2. Summary of cell tropism of Rickettsiales species *in vivo* and *in vitro*.

★ = *in vitro* growth

▼ = *in vivo*

1.5. Peptidoglycan

1.5.a. The bacterial cell envelope

Bacteria are surrounded by a cell envelope. In Gram positive bacteria, this consists of an inner lipid membrane with a rigid cell wall. However, Gram negative bacteria also have an outer lipid membrane which surrounds the periplasm [159]. Peptidoglycan, also known as murein, is an essential component of the cell wall of most bacterial species. It is located outside the plasma membrane and its structure can help to differentiate Gram negative and Gram-positive bacteria by the Gram-staining method. Gram positive bacteria have multiple sheets of peptidoglycan in their cell wall whereas Gram negative bacteria have only a few layers in the periplasm which is protected by the outer membrane [160].

Bacteria have many important components in their cell envelope. The surface layer, or S-layer, is found on the surface of most bacteria and is composed of a hexagonal lattice of identical subunits of glycoproteins known as surface layer proteins (SLPs). These contain some of the most abundant proteins of the bacteria and the biogenesis of these proteins takes up most of the cell's metabolic sources [161]. In Gram positive bacteria, the S-layer attaches to the peptidoglycan whereas in Gram negative bacteria, the S-layer attaches directly to the components of the outer membrane, such as the LPS [162]. The S-layer has pores which are thought to act like a molecular sieve and can contribute to virulence. In *R. typhi* and *R. prowazekii*, proteins of the S-layer act as immunogens and are potential vaccine targets [163].

Lipopolysaccharide (LPS) makes up the outer leaflet of the outer membrane in Gram-negative bacteria. LPS is a large macromolecule and is made up of hydrophobic and toxic lipid A, the hydrophilic core polysaccharide and a repeating hydrophilic o-antigenic polysaccharide side chain. The LPS is immuno-stimulatory and is anchored to the outer membrane by lipid A [164].

Like peptidoglycan, LPS is assembled at the plasma or inner membrane. As a pathogen associated molecular pattern (PAMP), Lipid A is responsible for the toxicity of the bacteria, as lysed LPS fragments of bacteria are recognised by host Toll-like receptor 4 which may lead to diarrhea, fever and septic shock. LPS is essential to the structural integrity of the cell wall and increases the negative charge of the structure to the phosphate groups at its base. LPS also provides protection against chemical attacks and certain antibiotics [165].

1.5.b. The role of peptidoglycan

Peptidoglycan has 3 major roles in bacteria. These roles are growth, division and providing protection from osmotic pressure [166].

Peptidoglycan is needed for cell growth and division to occur. Although its biosynthesis is similar in all bacteria, its regulation determines the bacterial cell shape [1] [167]. Peptidoglycan synthesis occurs at the septum and the lateral wall, serving as a scaffold for components of the cell envelope, such as proteins and teichoic acids. The peptidoglycan synthesis machine in these locations interact with the cytoskeletal proteins MreB and FtsZ [168].

Cell division is a tightly coordinated event in bacteria. Peptidoglycan interacts with the bacterial tubulin protein, FtsZ. This GTPase protein polymerises into single stranded filaments which form the z ring at mid cell. This ring marks the future site of division at the septum and is maintained by the treadmilling of FtsZ polymerisation. The speed of treadmilling regulates the rate of peptidoglycan synthesis and cell division, which differs among bacterial species [169]. FtsZ recruits the divisome, a large protein complex, and with the help of septal transpeptidase penicillin binding proteins and amidases, the bacterial cell can divide into two daughter cells. The septa then becomes the old poles of the bacteria and peptidoglycan is unable to be synthesised there [170].

MreB regulates new peptidoglycan insertion to allow the cell wall to expand for growth. The actin homolog MreB forms filaments which localise to the outer cell wall in distinct patches, of most rod-shaped bacteria. During elongation, MreB interacts with components of the cell wall synthesis machinery, such as class A penicillin binding proteins, PBP1A and PBP2 [166] [171]. MreB may also form complexes with the precursor proteins such as MurA-MurG and MraY. As well as controlling peptidoglycan synthesis, MreB is also involved in the determination of cell shape as it has been shown to interact with integral membrane protein RodA and RodZ [172].

MreB was thought to exist as a helical filament around the periphery of the cell but due to advancements in microscopy (deconvolution microscopy or total internal reflection fluorescence microscopy, TIRFM), recent research presents MreB as rotating around the long axis of the cell in a bidirectional manner [173]. This rotation may depend on the presence of peptidoglycan synthesis machinery [174]. When MreB is inhibited, the cell wall may grow but the diameter is no longer regulated.

Lastly peptidoglycan maintains the turgidity of the cell and balances the internal osmotic pressure [175]. A decrease in external osmotic pressure causes water influx which may lead to lysis [176]. The peptidoglycan structure provides the mechanical strength to resist high intracellular turgor.

Peptidoglycan in bacteria can also be responsible for its destruction. It is recognised by the host as a pathogen-associated molecule pattern molecule (PAMP), alerting innate immune cells through Toll-like receptors to invading pathogens [177]. This is particularly dangerous to obligate intracellular bacteria such as the Rickettsiales, some of which exist free in the cytoplasm. This may explain why many of these bacteria have very little or lack completely peptidoglycan. The presence of peptidoglycan is also used as a drug target. Beta-lactam antibiotics such as penicillin binds with penicillin binding proteins (DD-transpeptidases) to hinder the production of new peptidoglycan. Lysozyme, naturally found in the innate immune system, is able to hydrolyse the 1,4 beta glycosidic bond between polysaccharide residues [178].

1.5.c. The structure of peptidoglycan

Peptidoglycan is composed of linear glycan sheets, cross-linked by short peptides. The glycan strands consist of alternating N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) residues which are linked by 1,4 beta glycoside bond [166] (**Figure 6**). Attached to MurNAc are short peptide side chains, consisting of L-alanine, D-glutamate, *meso*-diaminopimelic acid (*meso*DAP) and two D-alanines. MurNAc, an unusual sugar, *meso*DAP and the D-amino acids, are unique to the bacterial cell wall. However the structure of peptidoglycan such as the organization of the length of the glycan chains and the order of the amino acids in the peptide chain can vary among bacterial species [1, 179].

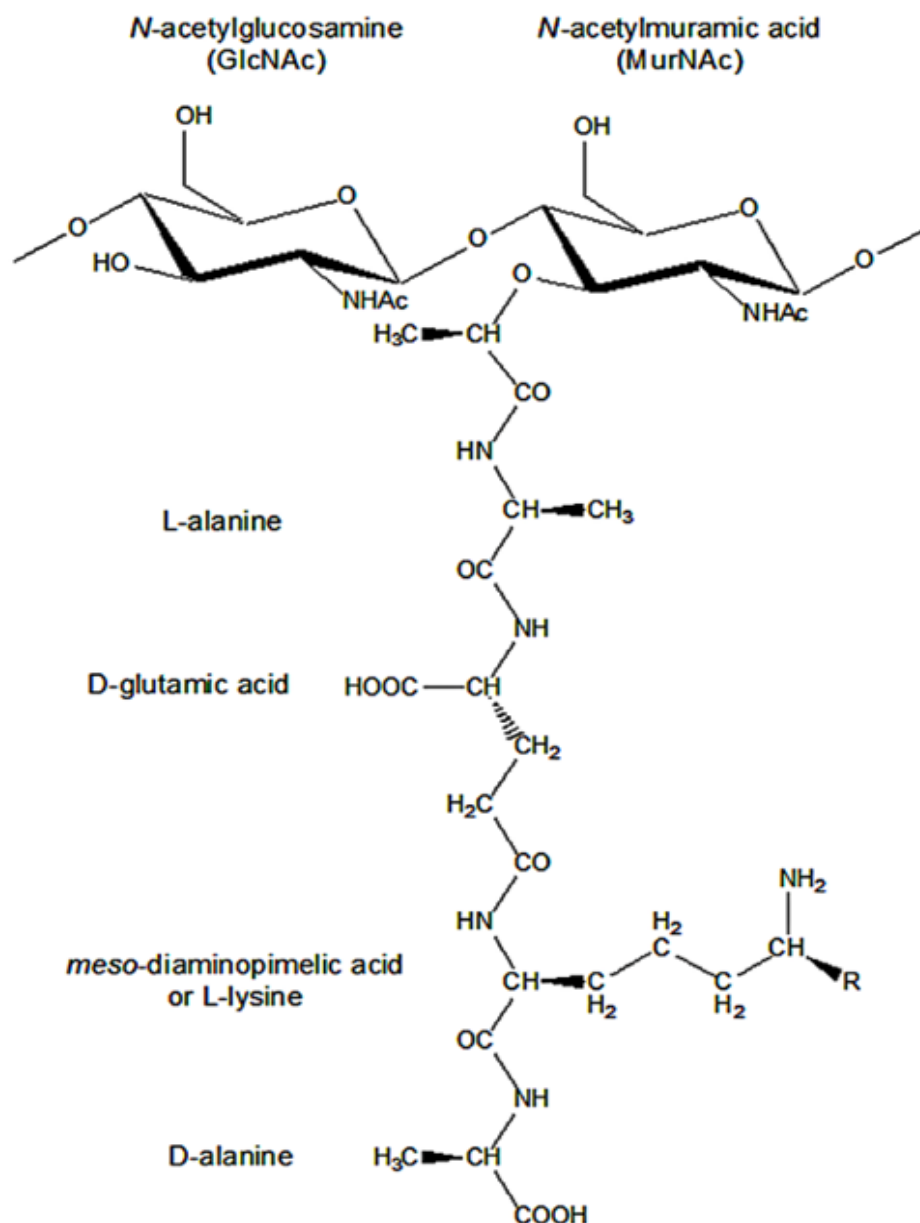


Figure 6. The molecular structure of a monomer subunit of peptidoglycan.

The disaccharide back bone of lipid II is made up of *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic (MurNAc) linked by a β -1,4 glycosidic bond. In Gram negative bacteria, the peptide chain at position 3 is meso-diaminopimelic acid but L-lysine in Gram positive bacteria. [180]

Peptidoglycan synthesis begins in the cytoplasm of the bacteria, where UDP-MurNac and the pentapeptide is produced, aided by enzymes in the Mur pathway and Ddl ligase. This precursor is ligated to undecaprenyl polyprenyl phosphate (C55PP) a universal carrier lipid, by MraY, which results in the formation of Lipid I. Now in the bacterial membrane, lipid I progresses to lipid II by the addition of a GlcNac residue which is catalyzed by MurG. This lipid precursor can then be transported over the inner membrane by flippase for the final steps of peptidoglycan synthesis. Lipid II can then be polymerised into the glycan strands by a transglycosylation reaction due to the glycosyltransferase activity of PBPs [181]. The polysaccharide strands which result from this transglycosylation are covalently linked by the transpeptidation activity of PBPs. This leaves a peptide bond which is able to crosslink between the glycan strands. This crosslinkage occurs between mesoDAP and D-alanine in *Orientia*, by DD-transpeptidases [166, 182].

1.5.d. Penicillin Binding Proteins (PBPs)

Penicillin binding proteins are named after the enzymes which bind to the antibiotic, as found by Alexander Fleming in 1928, who noticed penicillium growing on his bacterial culture plates. As it can mimic the ligands for enzymes which are crucial for peptidoglycan synthesis, the binding of penicillin prevents growth of bacteria. The beta-lactam ring of penicillin is able to covalently bind to active site serine residue of DD-transpeptidase PBPs, blocking the active site by acylation. Bacteria have developed resistance to the behavior of beta-lactam antibiotics by producing beta-lactamase. By hydrolysis of the cyclic amide bond, beta-lactamase inactivates the beta-lactam ring [183] [184].

PBPs exhibit two activities in peptidoglycan synthesis. They may catalyse the polymerization of the glycan strands in a transglycosylation reaction, between GlcNac and MurNac groups. The

second activity is the crosslinking of the peptide chains between glycan strands in a transpeptidation reaction [184]. The transpeptidase must recognize the terminal double d-Ala residue of the peptide chain and catalyse the reaction between the carbonyl group of d-Ala and the adjacent amino at position 3 of the alternate peptide chain to create the new amide bond, which is most likely to be mesoDAP. These links are the targets of β -lactams. This DD-transpeptidase D-Ala⁴-mesoDAP³ crosslink is common among bacterial species but can alter depending on the bacterial species due to structural variation in their peptidoglycan structure. β -lactams can mimic the double D-alanine residues on the peptide chain and inactivate the DD-transpeptidase completely. The reverse of these activities may also be carried out through endopeptidation and transpeptidation [185].

However, crosslinks can also form between the 3rd residues of the peptide chains by LD-transpeptidase activity. These crosslinks are not generated by PBPs but are catalysed by LD-transpeptidases instead, between the two mesoDAP residues. These LD-transpeptidases have been identified as the main crosslinking enzyme in Gram positive and negative bacteria such as *Enterococcus faecium*, *Mycobacterium tuberculosis*, *Clostridium difficile* and *E. coli* [186] [187] [188]. Also, structurally different, instead of a serine active site, LD-transpeptidases utilise a cysteine active site and the crosslink created is therefore resistant to β -lactams. There have been reports of antibiotics from the carbapenem class such as imipenem having an effect on the LD-transpeptidases, so bacterial species such as drug-resistant strains of *Mycobacterium tuberculosis* may be treated [189]. LD-transpeptidases are also able to transfer Braun's lipoprotein on to mesoDAP [190].

Penicillin binding proteins are divided into two classes based on their molecular mass by SDS-PAGE results based on *E.coli* [185]: high and low molecular mass. High molecular mass PBPs are divided into class A and class B. Class A PBPs are bifunctional, as they possess an extracellular domain which confers glycosyltransferase 51 activity and a c-terminal domain which

catalyses transpeptidation activity. To achieve both activities, the class A PBPs interact with two conserved machines, the Rod complex and the divisome [191].

Class A have 7 subclasses: A1-A2 consist of Gram-negative PBPs, A3-A5 for Gram-positive. A6 are PBPs which are unusual as they are not necessarily needed. Class B PBPs on the other hand, are only monofunctional, catalyzing the transpeptidation reaction between the peptide chains as they consist of a C-terminal transpeptidase penicillin binding domain. The N-terminal does not have any catalytic activity and is thought to interact with proteins involved in the cell cycle. Class B have 5 subclasses and are vital for resistance against beta-lactam drugs [192].

Low molecular mass PBPs consist of just 1 group, C (c1, c2 and c3). They have a penicillin binding domain and may also have either DD-carboxypeptidase or DD-endopeptidase activity.

Orientia does not have the genes which encode class A PBPs, which is the PBP normally responsible for glycosyltransferase activity. The glycan chains of the peptidoglycan must be polymerised through another enzymatic activity. This is also the case for *Wolbachia*, as researchers have hypothesized that a classic peptidoglycan structure is not present but unpolymerised lipid 2 precursors may instead form a peptidoglycan-like structure [193].

1.5.e. Peptidoglycan and SEDS

Peptidoglycan synthesis requires the coordination of multi-protein complexes. The Rod complex, also known as the elongasome, drives cell elongation by moving in a circumferential manner around the cell axis and is dependent on peptidoglycan synthesis. The divisome synthesizes peptidoglycan during cytokinesis. Both the ROD and the divisome interact with PBPs during peptidoglycan synthesis. They also interact with the RodA/FtsW proteins from the SEDS family (shape, elongation, division and sporulation) during lipid II exportation and interact with Mra/Mur proteins for lipid II synthesis [181] [194]

RodA and FtsW are integral membrane proteins, responsible for cell growth and cell division respectively [195]. There is recent evidence suggesting that the SEDS proteins might perform glycosyltransferase activity, since they are structurally similar to membrane anchored glycosyltransferases [181]. This indicates they may interact with monofunctional transpeptidases such as FtsI (PBP3) and PBP2. By fluorescence colocalization microscopy it is known that FtsW interacts with FtsI at the septum and RodA interacts with PBP2 along the cell body [196] [195]. These partnerships would act as bifunctional PBPs. Some bacterial species are known to be missing class A PBPs such as *Orientia*, *Wolbachia* and *Chlamydia*, so a SEDS-class B PBPs partnership could explain how these bacterial species polymerise their glycan chains [197].

1.3.f. Immune response to peptidoglycan

As a PAMP, peptidoglycan is recognised by the host immune system. Bacteria activate the innate immune response upon entry into host cells. This involves recognition by intracellular nucleotide-binding oligomerisation domain containing proteins (NOD) 1 and 2 [198]. Also, pyrin domain-containing 3 (NLRP3) and peptidoglycan recognition protein 1 (PGLYRP1) and toll-receptor 2 are activated [199]. These all trigger inflammatory responses in the host.

The nucleotide-binding oligomerisation domain (NOD) proteins NOD1 and NOD2, the founding members of the intracellular NOD-like receptor family, sense conserved motifs in bacterial cell wall peptidoglycan and induce pro-inflammatory and antimicrobial responses [198]. NOD1 receptor recognises peptidoglycan containing meso-diaminopimelic acid (*meso*DAP) which is unique to Gram negative bacteria [200]. NOD1 has the ability to activate downstream signalling such as mitogen activating kinases which leads to inflammation due to pro-inflammatory cytokines such as IL-32 in *Orientia* [201]. This induces a whole host of other cytokines [202]. NOD2 recognises muramyl dipeptide (MDP) which subsequently activates NF- κ B and MAPK pathways

in the host cells, leading to the up-regulation of transcription of pro-inflammatory genes [200] [203].

The secretion of IL-32 is significant because it is found in epithelial cells and regulates (with IL- β) the cells inflammation by influencing the expression of cellular adhesion molecules 1 (ICAM-1) outside the cell. When an endothelial cell becomes infected, IL-1 and TNF α are released and upregulate ICAM-1 as well as p-selectin, e-selectin and VCAM-1. This all promotes cellular influx and IL-6 and IL-8 initiate the inflammatory response. Therefore, the NOD1-IL-32 pathway is a modulator of the immune response in endothelial cells to *Orientia*. After being infected with the bacteria, the immune response consists of inflammatory cytokines such as TNF- α IL-1 β and IL-6 which cause high fever and then become reduced after antibiotic treatment [201].

Chapter 2

Materials and Methods

2.1 Eukaryotic cell culture

For all *Orientia* experiments, L929 mouse fibroblast cells were used. Mouse fibroblast L929 cells (ATCC CCL-1), were provided by Prof Stuart Blacksell at the Mahidol Oxford Tropical Research Unit in Bangkok, Thailand. L929 cells were grown in RPMI 1640 Medium with HEPES (Thermo Fisher Scientific, 22-400-071, USA) supplemented with 10% heat inactivated FBS (Thermo Fisher Scientific, 16140071, USA) in 25cm² flasks at 35°C and 5% CO₂.

Kidney epithelial Vero cells (ATCC CCL-81) were provided by Dr Nancy Connell at PHRI, Rutgers, New Jersey, USA. Vero cells were grown in RPMI 1640 Medium with HEPES, supplemented with 10% heat inactivated FBS in 25cm² flasks at 37°C and 5% CO₂.

HEK-Blue™ hNOD1 (Invivogen, chkb-hnod1, USA) cells were taken from human embryonic kidney cells. These cells were grown in 25cm² flasks with growth medium DMEM (Thermo Fisher Scientific, 21013, USA) with 4.5 g/l glucose, 10% fetal bovine serum, 100 U/ml penicillin, 100 mg/ml streptomycin, 100 mg/ml Normocin™ and 2 mM L-glutamine. Selective antibiotics, 30 µg/ml of blasticidin and 100 µg/ml of Zeocin™ were added after passage 2 to maintain the cell line.

Macrophage-like DH82 cells (ATCC CRL-10389) were grown in 25cm² flasks with Eagle's Minimum Essential Medium (EMEM) (Sigma, M0325, USA) with 10% heat inactivated FBS at 37°C and 5% CO₂.

Human leukemia HL-60 cells (ATCC CCL-240) were grown in 25cm² flasks with Iscove's Modified Dulbecco's Medium (IMDM) (ATCC 30-2005) with 10% heat inactivated FBS at 37°C and 5% CO₂.

Aedes albopictus clone C6/36 cells (ATCC CRL-1660) were grown in 25cm² flasks with Eagle's Minimum Essential Medium (EMEM) (Sigma, M0325, USA) with 10% heat inactivated FBS at 28°C and 5% CO₂.

Cell lines were cultured every two or three days and seeded on slides for infection for fixed imaging two days previous and for live imaging, one day previously.

2.2 Bacterial propagation

The Karp-like strain UT76 was used for all *Orientia* experiments. Bacteria were grown in L929 cells in 25cm² flasks at 35°C and 5% CO₂ using RPMI 1640 Medium with HEPES and 10% FBS. Bacteria were taken from day 7 infections inside host cells. This involved scraping host cells and lysing them by force using a bullet blender (NextAdvance, USA) at power 8 for 1 minute. The host cell debris was removed by spinning bacteria suspension at 400rcf for 5 minutes. Bacteria were then filtered through a 2.0µm membrane filter and spun down at 14500rpm for 5 minutes to be resuspended into fresh media or buffer. The full protocol can be found here [138].

R. canadensis, *A. marginale*, *Wolbachia*, *A. phagocytophilum* and *E. chaffeensis* were isolated 3-7 days' post infection from 6 well plates in 37°C or 28°C (only C6/36 cells) and 5% CO₂. *R. canadensis* were taken from intracellular host cells by lysing scrapped host cells with 0.2mm beads (Thomas scientific, 11079101, USA) for 1 minute by vortex, using the same protocol as previously documented for *Orientia*. Host cell debris was removed by spinning bacteria suspension at 400rcf for 5 minutes.

A. marginale, *Wolbachia*, *A. phagocytophilum* and *E. chaffeensis* bacteria were taken from extracellular media. This was pelleted at 145000rpm, with the media then discarded. The bacterial pellet was then resuspended in culture media.

E. coli and *B. subtilis* were grown in LB media at 37°C and 5% CO₂ until log phase was reached.

2.3 Immunofluorescence labelling

Cells were fixed with 4% paraformaldehyde (PFA) (Thermo Fisher Scientific, 28906, USA) for 10 minutes at room temperature. Other concentrations of paraformaldehyde were not used or tested. After fixation, slides were washed with 3X PBS and then permeabilised with 0.5% triton X100 (Sigma, X100, USA) for 10 minutes. After additional washes with PBS, the primary antibody was added to cells (dilution dependent on the antibody) for 30 minutes at 37°C and 5% CO₂. Excess primary antibody was washed off with 3X PBS wash and a corresponding fluorescent secondary antibody was added for 30 minutes at 37°C and 5% CO₂. Finally, after a 3X PBS wash, cell were coated with VECTASHIELD® Antifade Mounting Medium with DAPI (Vector Laboratories, H-1200, USA) or Hoechst (Thermo Fisher Scientific, 1399, USA) at 1 µg/ml was added to cells for 10 minutes at room temperature before the addition of mounting media.

2.4 CFSE/CellTrace FarRed labelling

CFSE (Thermo Fisher Scientific, C34554, USA) and CellTrace FarRed (Thermo Fisher Scientific, C34572, USA) were dissolved in DMSO in 5µM aliquots and stored at -20°C. One aliquot was thawed at room temperature and then diluted to 5µM in PBS with 2% Invitrogen™ UltraPure BSA (Invitrogen, AM2616, USA). Purified bacteria were taken from lysed host cells and centrifuged at 14500rpm for 5 minutes at room temperature. Bacteria were then resuspended in the 5µM dye solution for 15 minutes at 37°C and 5% CO₂ or 15 minutes at room temperature, in the dark. Bacteria were then centrifuged at 14500rpm for 5 minutes at room temperature twice to remove residual dye. After washes, bacteria were finally resuspended in media containing 10% FBS and left for 20-30 minutes to allow the dye to quench before live imaging.

2.5 CFSE quantification

CFSE-labelled bacteria were added to L929 cells seeded on to Ibidi chamber slides (Ibidi, USA). Bacterial infection was imaged every day over 7 days by confocal microscopy, using the same laser strength and parameters for FITC imaging each time. However, as a comparison, the FITC strength was also increased to show weakly labelled bacteria. Alongside this, CFSE-labelled *Orientia* were also kept at 37°C in culture media in an Ibidi slide also, with no host cells. This was imaged once a day for 7 days also.

CFSE labelling over time was quantified using ImageJ [204]. For each time-point, 10 bacteria were chosen at random and the brightest pixel in each bacterium was documented for intensity. The intensity figures were plotted on GraphPad Prism (GraphPad Software, La Jolla California USA, www.graphpad.com). This experiment was completed more than three times and every result presented the same conclusion.

2.6 Syto9 labelling

Syto9 (Invitrogen, S34854, USA) was dissolved in DMSO in 5µl aliquots at 5µM, stored in 20°C. The dye was added to media at 5nM and added to infected host cells. The dye was incubated for 20-30mins at 37°C to allow sufficient labelling. Following this, the media plus the dye was washed off host cells and fresh media was added before confocal imaging.

2.7 HADA/HALA labelling

HADA/HALA were kept in aliquots at -20°C and was added to isolated bacteria in fresh culture media at 1mM. Bacteria were incubated at 37°C and 5% CO₂ for 3 hours and then washed with 3X PBS. Bacteria were then imaged live by confocal microscopy or fixed with 4% PFA for 10 minutes at room temperature.

2.8 Vybrant labelling

Vybrant dyes, DID, DIO and DIL (LifeTechnologies, V22889, USA) were used to label isolated bacteria. Dyes were kept in aliquots at 4°C and used at a final concentration of 100µM to label bacteria in 100µl of Opti-MEM buffer (ThermoFisher Scientific, 31985062, UK). Bacteria were incubated with the dye at 37°C for 30 minutes. After, bacteria were pelleted at 14500rpm to wash off residual dye and washed in 2X PBS. Following the final wash, bacteria were resuspended in culture media and then added to host cells for infection and imaging.

2.9 Click chemistry

The azide or alkyne probe was added to infected cells for a specific duration of time. Following this, cells were fixed with 4% PFA for 10 minutes at room temperature. After fixation, cells were washed with 3X PBS and permeabilised with 0.5% triton X100 for 10 minutes. Click chemistry was performed using the Click-iT™ Cell Reaction Buffer Kit (Invitrogen, C10269, USA) with an azide or alkyne Alexa Fluor 488/594 (AF 594 Alkyne, 1297-1 or AF 488 Picolyl Azide 1276-5, Click Chemistry Tools, USA) for 1 hour in the dark. After 3X PBS washes, cells were either further labelled with antibodies or Hoechst.

2.10 EDA/EDA-DA labelling

EDA (Alfa Aesar, H65798, USA) and EDA-DA (produced in-house by Professor Arkady Mustaev) were diluted in water and used at 1mM. Probes were added to infected host cells and incubated for various times at 37°C and 5% CO₂ and washed with 3X PBS to then be fixed with 4% PFA for 10 minutes. Incubation times for each species were: *B. subtilis* and *E. coli* for 10 minutes, *R. canadensis* for 30 minutes, *A. marginale*, *A. phagocytophilum*, *Orientia*, *Wolbachia* and *Ehrlichia* for 4 hours. In drug sensitivity assays, EDA and drug were added simultaneously for the same duration of time. After fixation, click chemistry was performed. For *Orientia* imaging, antibody labelling using TSA56 followed click labelling, with the secondary antibody, goat anti-Rat IgG (H+L) Cross-Absorbed Secondary Antibody, Alexa Fluor 594 (Thermo Fisher Scientific, A-11007, USA). Hoechst at 1 µg/ml was added to cells for 10 minutes at room temperature before imaging.

2.11 RNA FISH labelling

Infection was fixed at certain time-points with 4% PFA. After washing with 3X PBS, 70% of ethanol was added to cells and incubated for 1 hour on ice. Cells were then washed twice with PBS and permeabilised with 0.5% triton X100 for 30 minutes on ice. Cells were washed 2X PBS and 1mg/ml lysozyme in TE buffer was then added for 1 hour at room temperature. BSA (Invitrogen, AM2616, USA). blocking was then carried at 1mg/ml in PBS for 30 minutes at room temperature. After another PBS wash, 2x UltraPure SSC (Thermo Fisher Scientific, 15-557-044, USA) plus 10% formamide (Sigma, S4117, USA) was added for 1 minute to equilibrate cells. The 16s probe (produced by Suparat Giengham) was produced from 33 oligo sequences of the 16s gene. The prob was used at 1ng/µl and diluted in hybridisation buffer (produced in-house), containing 10% dextran sulfate (Sigma, 265152, USA), 2 mM vanadyl-ribonucleoside complex (Sigma, 94742, USA), 0.02% RNase-free BSA (Sigma, A9647, USA), 50 µg *E. coli* tRNA (Sigma, 10109541001, USA), 2x UltraPure SSC (Thermo Fisher Scientific, 15-557-044, USA) and 10% formamide

(Sigma, S4117, USA). This was added to cells for 5-6 hours at 50°C and was then further incubated at 37°C overnight. Cells were then incubated with 2x UltraPure SSC with 10% formamide for 5 minutes with gentle shaking, twice. In between incubations, cells were washed with 2x UltraPure SSC. DNA was labelled with 0.1 µg/ml Hoechst, for 10 minutes at room temperature. After 3X PBS washing, mounting media was added to the slide for imaging by confocal microscopy.

2.12 Methionine labelling

Probes Click-IT™ L-Homopropargylglycine (HPG) (Thermo Fisher Scientific, C10186, USA) or L-Azidohomoalanine (Click Chemistry Tools, 1066-25, USA) were added to live bacterial infections at 50 µM, in methionine free DMEM culture media (Thermo Fisher Scientific, 21013024, USA). After 30 minutes, the probe and culture media were discarded, and the cells were fixed with 4% PFA for 10 minutes at room temperature. After permeabilisation with 0.5% triton X100 for 10 minutes, click chemistry was performed. Following this, further labelling may have been carried out such as the addition of antibodies for confocal imaging.

2.13 Hot Shot DNA Extraction

Lysis buffer (25mM NaOH, 0.2mM EDTA) was added to cells. Cells were then placed in safe-lock 1.5ml tubes (VWR, 20901-547, USA) and placed in 90°C for 30 minutes. Neutralisation buffer (40mM Tris-HCl in ddH₂O) was then added to cells, in the equal volume at which lysis buffer was added. 1 µl of this volume was added to qPCR.

2.14 Quantification by qPCR

Orientia bacteria growth was tested by quantitative real time PCR. Labelled bacteria or mock treated bacteria were grown in 24 well plates (BC017, Corning USA) seeded with mammalian host cells in 1.9cm² wells. Each condition was done in triplicate. The plate was washed after 3 hours to remove extracellular bacteria. After 7 days of growth at 37°C and 5% CO₂ bacterial DNA was isolated using the hot-shot extraction method. The genome copy number was quantified using the TSA47 gene by qPCR using primers, F = TCCAGAATTAAATGAGAATTTAGGAC, R = TTAGTAATTACATCTCCAGGAGCAA and probe = TTCCACATTGTGCTGCAGATCCTTC.

Other Rickettsiales species were grown in various cell lines seeded on 96 well plates (VWR, 25382-342, USA). Bacteria were extracted from host cells using the hot-shot DNA extraction method, at multiple time-points. Growth was measured by qPCR using the 16s gene using primers, F = CCGTGAATACGTTCTCGG, R = AAGGAGGTAATCCAGCCGCA and probe = CTTGTACACACCGCCCGTC.

qPCR was performed using a Bio-Rad machine and Multiplate™ 96-Well PCR Plates (Bio-Rad, MLL9601, USA).

2.15 Mass spectrometry

Mass spectrometry was performed on lysed bacteria, which were taken from infected L929 host cells after 7 days. Bacteria were purified by lysing host cells by mechanical lysis and then filtered through a 2µm filter. Bacteria were pelleted at 14500rpm and washed several times using 600ul of ice-cold 300mM sucrose. After removing supernatant, the bacterial pellet was autoclaved and then hydrolysed with 4M HCl at 100°C for 16 hours. The amino acids were transformed into volatile N-heptafluorobutyryl-isobutylester derivatives using Protocol 10 of Schumann [205]. The

samples were then analysed by GC/MS (320 Singlequad, Varian) and diaminopimelic acid was identified as a gas chromatographic peak with a retention time of 22.2 min and a set of fragment ions at 380, 324, 306 and 278 m/z.

2.16 Gene expression of *Orientia* by qRT-PCR

L929 host cells were infected with UT76. At each time-point over the course of 7 days, infected cells were placed on ice and quickly resuspended in RNAProtect Bacteria Reagent (Qiagen, 76506, USA) and kept at -80°C. RNA extraction was carried out using Qiagen RNeasy Plus kit (Qiagen, 74136, USA), as recommended by the manufacturer's instructions. Purified RNA at 10µg, was treated with DNaseI (Thermo Fisher Scientific, AM2238, USA) at 37°C for 30–60 min. DNaseI-treated RNA was then converted to cDNA using the iScript reverse transcription supermix (Biorad, 170–8841, USA) with random primers. A reverse transcriptase-free control reaction was performed to verify no genomic DNA remained to contaminate the RNA sample. cDNA was then stored at -20°C until used as a template for qPCR, using gene specific primers for *mipZ*, *murA*, *murD*, *murF*, *ddl* and *pbp2*. qPCR was carried out using the SYBR green qPCR mix (Biotools, Houston, USA, catalog number 10.609) and housekeeping gene *mipz* normalised expression levels.

2.17 Drug sensitivity

UT76 were grown in 24 well plates seeded with L929 cells. Various drugs were added to the plate after infection apart from lysozyme and mutanolysin which cannot enter mammalian cells. For lysozyme treatment, bacteria were resuspended in SPG with and without lysozyme, for 15 minutes at 37°C before being added to L929 cells. For mutanolysin treatment, bacteria were resuspended in media with and without mutanolysin for 15 minutes at 37°C before being added to L929 cells. Growth was detected after 7 days by quantification of bacteria by qPCR. Alongside

this, bacteria were infected in L929 cells seeded on Ibidi chamber slides (Ibidi, USA) and treated with drugs for 24 hours.

All other Rickettsiales species were grown in 96 well plates in various cell lines. Drugs chloramphenicol, penicillin, phosphomycin and d-cycloserine were added to cells 1 hour after infection. Growth of bacteria were measured by qPCR 4 days after infection. Only phosphomycin was added to infected Ibidi slides for imaging.

The drug concentrations used were: chloramphenicol 100 ug/ml; penicillin G 150 ug/ml; D-cycloserine 250 ug/ml; phosphomycin 40 ug/ml; EDTA 1 mM, lysozyme 5 ug/ml; mutanolysin 80 ug/ml; imipenem 10 ug/ml; meropenem 10 ug/ml; mecillinam 2 mg/ml; tazobactam 100 ug/ml.

2.18 Western blot analysis

Infected or uninfected L929 cells were mechanically lysed in a Bullet Blender (Next Advance, USA), at power 8 for 1 min. Cells were pelleted, washed with PBS 1/2 times and resuspended in a small volume of PBS. The protein concentration was measured by nanodrop analysis and the sample volume was adjusted to guarantee equal loading. Samples were mixed with SDS-PAGE buffer with or without 20% β -mercaptoethanol (Sigma, M6250, USA) and samples were then loaded on 12% Mini-PROTEAN TGX Precast Protein Gels (Bio-Rad, 4561043, USA). Monoclonal antibody TSA56 was used to compare western blots and probed with secondary antibody conjugated with alkaline phosphatase (Promega, S3831, USA). Western blot was developed using an alkaline phosphatase detection kit (Promega, USA, catalog number S3841).

2.19 DTT treatment of *Orientia*

Bacteria were purified from infected cells and then resuspended in cell culture media with and without 1mM DTT. Bacteria were incubated at room temperature for 20 minutes and then pelleted and washed twice before being resuspended in fresh cell culture media. Bacteria were then added

to L929 cells seeded on Ibidi slides (Ibidi, USA) for 24 hours. After infection time had passed, cells were fixed with 4% PFA and after antibody labelling, bacterial quantification was performed in 100 host cells manually to estimate infection numbers.

2.20 NOD1 activation in Hek blue cells

Hek-blue hNod1 (Invivogen, hkb-hnod1, USA) were grown in growth medium DMEM with 4.5 g/l glucose, 10% heat inactivated fetal bovine serum, 100 U/ml penicillin, 100 mg/ml streptomycin, 100 mg/ml Normocin™ and 2 mM L-glutamine, plus selective antibiotics 30 µg/ml of blasticidin and 100 µg/ml of Zeocin. Cells were seeded on clear bottom black 96 well plates (Corning, 29444-008, USA) 2 days before infection. Bacteria were taken from prepared aliquots kept at -80°C in sucrose phosphate glutamine (SPG). All bacteria were heat inactivated at 90°C for 30 minutes before being added to host cells. All infections were carried out in triplicate. After 3 days of infection, growth media was replaced with HEK-blue detection media (Invivogen, hb-det2, USA) for SEAP detection. The plate was further incubated at 37°C and 5% CO₂ and quantified by spectrophotometry (Synergy H1, Biotek) at 640nm from 6 hours of the addition of detection media. Results were added to GraphPad Prism (GraphPad Software, San Diego California USA) and a T-test was performed to compare bacterial SEAP levels to positive control *E. coli*.

2.21 Sensitivity of the cell wall to lysozyme and mutanolysin

A methionine probe, Click-IT™ L-Homopropargylglycine (HPG) (Thermo Fisher Scientific, C10186, USA) was added to infected host cells for 30 minutes. Bacteria were then isolated from host cells and resuspended in sucrose phosphate glutamine (SPG) with and without 5µg/ml lysozyme and 5µg/ml mutanolysin. Bacteria were treated for 10 minutes at 37°C. After this time, bacteria were pelleted at 14500rpm and then resuspended in 10ul of PBS which was added to 12 well glass slides. This was dried and then fixed with 4% paraformaldehyde (PFA) for 10 minutes at room temperature and then permeabilised with 0.5% triton X100 for 10 minutes. Bacterial labelling was completed with click chemistry labelling with the Invitrogen™ Molecular Probes™ Click-iT™ Cell Reaction Buffer Kit (C10269, Thermofisher, USA) including AF 488 Picolyl Azide (1276-5, Click Chemistry Tools, USA) for 1 hour at room temperature in the dark. Hoechst at 1 µg/ml was added to cells for 10 minutes at room temperature and the slide was then imaged by confocal microscopy.

2.22 Sensitivity of the cell wall to osmotic pressure

A methionine probe, Click-IT™ L-Homopropargylglycine (HPG) (Thermo Fisher Scientific, C10186, USA) was added to infected host cells for 30 minutes. Bacteria were then isolated from host cells and resuspended in sucrose phosphate glutamine (SPG), cell culture media RPMI (22-400-071, Thermo Fisher Scientific, USA) and water. Bacteria were treated for 10 minutes at 37°C. After this time, bacteria were pelleted at 14500rpm and then resuspended in 10ul of PBS which was added to 12 well glass slides. This was dried and then fixed with 4% paraformaldehyde (PFA) for 10 minutes at room temperature and then permeabilised with 0.5% triton X100 for 10 minutes. Bacterial labelling was completed with click chemistry labelling with the Invitrogen™ Molecular Probes™ Click-iT™ Cell Reaction Buffer Kit (C10269, Thermofisher, USA) including AF 488

Picolyl Azide (1276-5, Click Chemistry Tools, USA) for 1 hour at room temperature in the dark. Hoechst at 1 µg/ml was added to cells for 10 minutes at room temperature and the slide was then imaged by confocal microscopy.

2.23 Phylogenetics

Rickettsiales tree was constructed by assembling the FtsZ gene of all species into Clustal Omega (EMBL-EBI, <https://www.ebi.ac.uk/Tools/msa/clustalo/>).

2.24 Microscopy

All imaging was performed using confocal microscopes, Zeiss LSM700 (Thailand) or Lecia DMi8 (USA). Mounting media for microscopy was produced in-house (20mM Tris, pH 8.0, 0.5% N-propyl-gallate and 90% glycerol).

Chapter 3

Labelling *Orientia tsutsugamushi*

Most of this research has been published: Live imaging of the genetically intractable obligate intracellular bacteria *Orientia tsutsugamushi* using a panel of fluorescent dyes, Atwal S, Giengkam S, VanNieuwenhze M, Salje J. 2016. J Microbiol Methods

3.1 Introduction

Due to the current lack of tools for genetic manipulation of *Orientia*, the use of genomically encoded fluorescent proteins for light microscopy of this organism is not possible [206]. Therefore, alternative strategies were developed to label *Orientia* for light microscopy imaging of both fixed and live samples. Two approaches were pursued: (i) generating a panel of *Orientia*-specific antibodies and (ii) optimising the use of a range of chemical probes and dyes.

3.1.a General principles of sample preservation for microscopy

Whether to image in real time or use fixed samples depends on the research question being answered. Live imaging allows dynamic cellular processes to be captured in real time, with less chance of experimental artifacts associated with fixation. In this case, bacteria can be tracked through their infection cycle over time in their native environment. However, this poses many problems, such as maintaining cells in their natural physiological state with regulation to pH, temperature and osmolality. Additionally, specifically labelling cells and keeping the toxicity to the minimum is very difficult over time [207].

Conversely, preserving cells using a fixative allows cells to be captured at a certain time point and the option of permeabilisation allows large molecules to enter for immunofluorescence labelling. IFM allows the detection of protein in cells which are in low abundance. The downside with using fixed cells though is that protein conformations tends to change depending on the fixative used and artifacts or false labelling is possibly leading to misleading conclusions [208].

For immunofluorescence microscopy to be successful, certain stages must be carried out [209]. This involves fixation, permeabilisation and labelling. Aldehyde fixatives crosslink proteins through free amino groups. Dehydrating and organic fixatives such as alcohols and acetone remove lipids and water from the cell resulting in the denaturation of the cellular macromolecules [210]. Permeabilisation must occur after aldehyde fixation to allow antibodies reach intracellular or intra-organellar antigens. The cell wall must be broken down to allow antibodies to pass through, but not completely broken down that the cell wall loses its structure. Triton x-100 is a non-ionic detergent with uncharged hydrophilic head groups which disrupts the hydrogen bonding in lipid bilayers as it demolishes the integrity of the membrane [211] [212].

3.1.b Selecting antigens for antibody generation

Antibodies were generated against surface proteins of *Orientia* because these would be easy to access for large antibodies, and because the main surface proteins of *Orientia* have been identified and are known to play roles in cell entry and immune stimulation.

Bacterial interactions into nonphagocytic cells is mediated by surface components on the bacteria and receptors on the host. At least 5 major surface antigens of *Orientia* have been identified from patient sera by western blot analysis [36]. Most of their functions are still unknown but most likely immunostimulatory and involved in bacterial entry.

Monoclonal and polyclonal antibodies can be generated commercially. Polyclonal antibodies recognise multiple epitopes on one antigen whereas monoclonal antibodies are more specific, detecting one epitope on an antigen. Polyclonal antibodies are produced when a specific antigen is injected into an animal and B cells are then activated. These B cells produce the polyclonal antibodies specific to the antigen. However, each B cell may respond to a different epitope on the antigen so that the resulting antiserum taken from the animal results in a range of antibodies which will target not one epitope but multiple. This is less specific but much cheaper to generate.

To have a more specific antibody, monoclonal antibodies can instead be produced. Monoclonal antibodies arise from a specific antigen injected into the animal which produces antibody producing B cells. These are fused with myeloma cells resulting in a hybridoma cell line. This cell line is capable of unlimited growth and can produce the antibody continually when grown in culture [213]. Each culture of the hybridoma will produce the same identical antibody so there is no batch to batch variability, keeping experiments consistent. These antibodies are more expensive, but results are more consistent [214].

Antibodies were generated after selecting major surface proteins of *Orientia* which are implicated in their uptake into host cells and infection cycle.

3.1.c. Chemical dyes and probes for labeling cells

A number of fluorescent dyes and probes have been developed for labeling live and fixed cells. These typically target specific biological molecules such as the lipid bilayer, nucleic acids or peptidoglycan. Many have not been tested specifically for use in bacteria. The applicability of a range of commercially available dyes for labeling *Orientia* was tested.

A range of labels were tested to evaluate which could be used to image *Orientia* for live and fixed imaging over the course of the infection cycle. Labels which would allow tracking of bacteria at entry and during their infection cycle in host cells were chosen.

3.2 Labelling *Orientia*: Objectives

Objective 1: To generate and test antibodies specific for membrane proteins of *Orientia*

- Approach: To commercially generate antibodies for TSA56, TSA47, TSA22 and ScaC and test them by western blot analysis and immunofluorescence microscopy.
- Expected outcome: A collection of validated antibodies against specific proteins in *Orientia*.

Objective 2: To develop a toolkit of probes to label specific components of *Orientia* cells for use in live and fixed microscopy imaging

- Approach: To test the labelling specificity of the probes listed in Table 3 by live and fixed imaging and to determine their effect on bacterial viability by growth inhibition measurements. The following criteria will be used to assess each probe: simplicity of technique, photostability during live imaging, stability of probe, and effect of probe on bacterial viability.
- Expected outcome: a collection of characterized probes with validated protocols for labelling *Orientia*.

Probe	Target
Carboxyfluorescein succinimidyl ester (CFSE/CellTrace FarRed)	Primary amines (cytoplasmic proteins)
SYTO9	DNA
Vybrant DIO, DID, DIL	Lipid membranes
HCC amino D-alanine (HADA)	Peptidoglycan
Clickable Ethynyl-D-alanine (EDA)/ Ethynyl-D-alanyl-D-alanine (EDA-DA)	Peptidoglycan
Clickable methionine	Methionine in proteins
RNA fluorescence in situ hybridization (RNA FISH)	RNA transcripts

Table 3. List of probes to be tested in this chapter and their specific targets

3.3 Results

3.3.a Antibodies

Polyclonal and monoclonal antibodies were generated against major surface proteins of *Orientia*: TSA56, TSA47, TSA22 and ScaC. Human serum was also taken from patients infected with *Orientia* to use as a positive antibody control. These were tested by Western blot for specificity to surface protein antigens (**Figure 7**) and then used for immunofluorescence microscopy imaging (**Figure 8**). The antibodies produced against the major surface proteins of *Orientia* gave varying amounts of success. Time courses were completed over the duration of the infection cycle with each antibody to understand protein expression. This indicated changes in protein expression at different stages during the infection cycle (not included in this thesis).

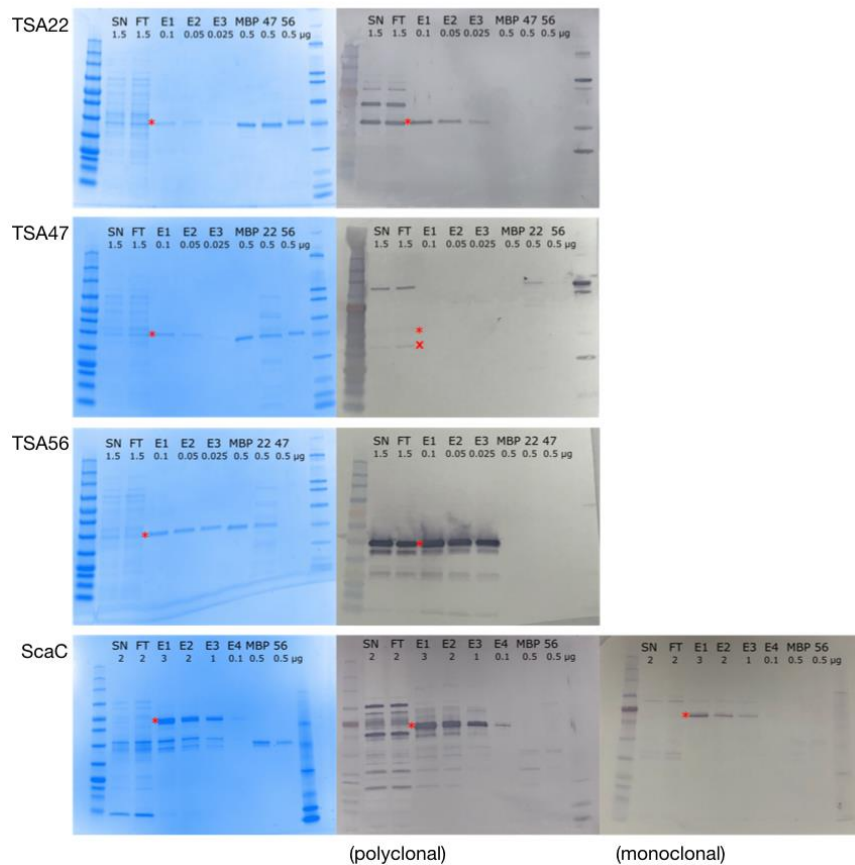


Figure 7. Specificity of antibodies against *Orientia*

Coomassie gel (left) and western blot (right) presenting the specificity of the antibodies produced against the surface proteins of *Orientia*. Maltose binding protein (MBP) was used as a protein expression tag and contained the peptide sequence for antibody generation and were purified here.

Work done by Jantana Wongsantichon.

SN= supernatant, FT = Flow-through, E1/2/3 = Elution number, MBP = pure maltose binding protein. 22, 47, 56 = purified peptide from TSA22, TSA47, TSA56 as negative controls.

Antibody name	Antigen	Protein sequence or Peptide	Adjuvant	Class	Vendor	Produced in
TSA56mono	TSA56	KLTPPQPTIMPIS IADRDF - KLH		Monoclonal	OxFab	Rat
TSA47mono	47kDa	KLH - TVQRGDRMLYIA LPNIN	Keyhole Limpet Haemocyanin (KLH)	Monoclonal	OxFab	Rat
TSA22kDa poly	22kDa	KLH - LKI KDFLISDDF		Polyclonal	OxFab	Rabbit
ScaC1 mono	ScaC	KSITPEKCKYPQ AEDIYEEDEEDE EDEDEEEEIADN DLLNVVSNMNL ISDMGEAREGLR KDIAEVSKLLCLL EQSTASSKNLTM RLQQSDIRETEQ SNYTGLLITEIESI NKENNDLQQTN NQLKIKLQQLEV KLEQALARNTDL QQI LPETRAEMDNL WKQNEQLKIQQLQ QLQVKLQNLPL QNKDGNAALINR AKLN	Keyhole Limpet Haemocyanin (KLH)	Monoclonal	GenScript	Mouse
ScaC2 poly	ScaC	KLH- KIQLQQQLQVTLQ NSLFLQNKNNNP A (JSA) + KLH - IAEVSKLLCMLE (JSB)	Keyhole Limpet Haemocyanin (KLH)	Polyclonal	OxFab	Rabbit
Human serum	Multiple			Polyclonal		Patients with scrub typhus

Table 4. Antibodies made against the surface proteins of *Orientia tsutsugamushi*.

Antibodies were produced by OxFabs, UK.

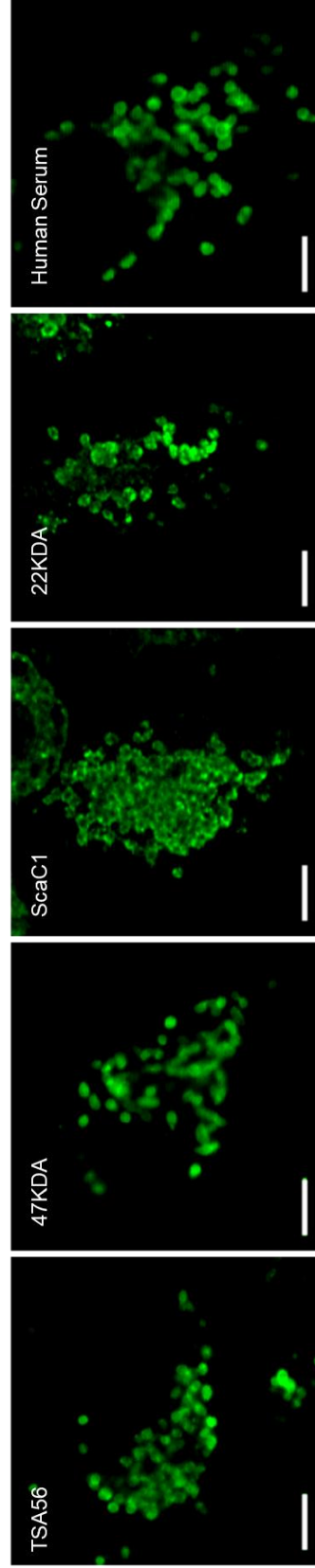


Figure 8. Intracellular *Orientia*, strain UT76, labelled with indicated antibody after fixation

L929 cells were infected with *Orientia* and during infection cells were fixed with paraformaldehyde (PFA) and then labelled with an antibody and imaged by confocal microscopy. Scale bar = 5µm

3.3.b CFSE

Bacterial entry is a rapid process. To follow this event, quick imaging is necessary but can be a challenge. Carboxyfluorescein succinimidyl ester (CFSE) (The CellTrace™ Cell Proliferation Kits, Molecular Probes USA) is a green fluorescent dye. CFSE is cell permeable and reacts with cellular proteins via covalent bonds with amine groups. Firstly the molecule carboxyfluorescein diacetate succinimidyl ester (CFDA-SE) diffuses into cells [215]. This molecule is lipophilic and not fluorescent. Intracellular esterases cleave two acetyl groups which produces the highly fluorescent CFSE, which covalently bonds with free amine groups on proteins. Once the acetyl groups are cleaved off, CFSE is impermeable and unable to leave the cell [216]. It is useful for live imaging over long periods of time as it is very photostable.

CFSE (emission 492, excitation 517) and CellTrace FarRed labelling (emission 630, excitation 661) with *Orientia* for the purpose of live imaging was tested. Both dyes were used at 5uM and presented cytoplasmic labelling at 0.5-2µm in diameter. The bacteria were labelled separately from host cells which resulted in no background or unspecific labelling once the bacteria infected cells.

The dyes are intended to be retained after aldehyde fixation. However high-quality labelling with CFSE or CellTrace FarRed after fixation was unsuccessful with acetone or paraformaldehyde. After fixation, fluorescence had clearly leached out from the bacteria and no specific bacterial shape by fluorescence was visible (**Figure 10**).

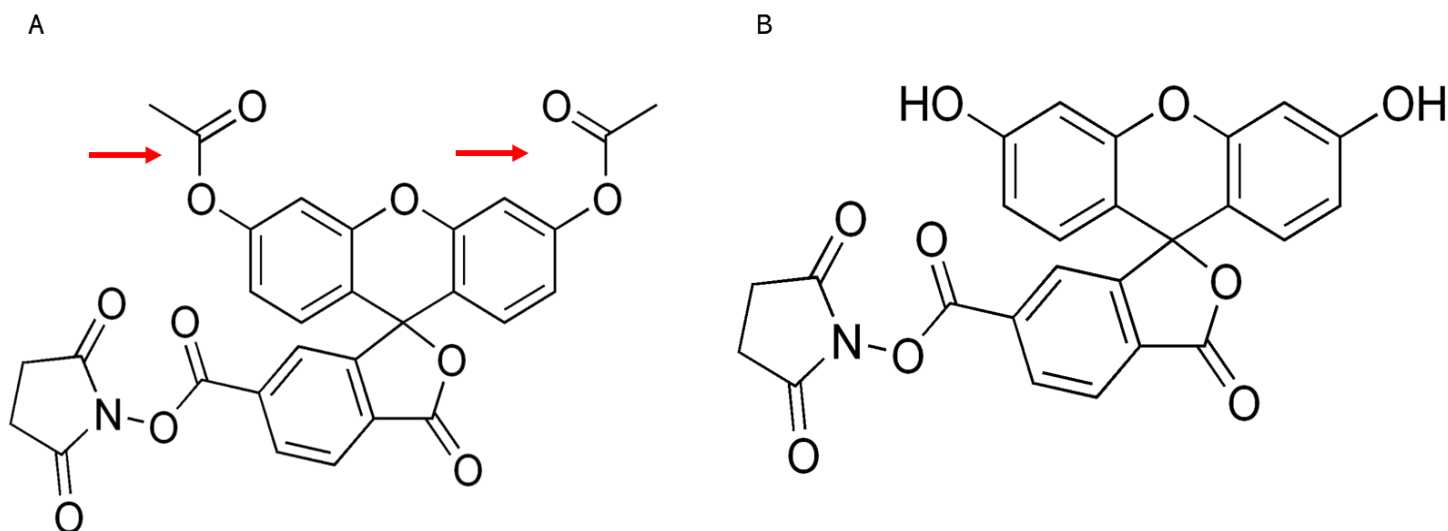


Figure 9. Fig: The molecular structure of cell staining dye CFDA-SE and CFSE.

A: carboxyfluorescein diacetate succinimidyl ester (CFDA-SE) with indicated acetate groups with red arrows, which render it highly permeable. Once inside the cell, esterases remove the acetate groups.

B: Carboxyfluorescein succinimidyl ester (CFSE) is the fluorescent product of the removal of acetate groups once inside the cell. (Images were taken from Wikipedia:https://en.wikipedia.org/wiki/Carboxyfluorescein_succinimidyl_ester, https://en.wikipedia.org/wiki/Carboxyfluorescein_diacetate_succinimidyl_ester)

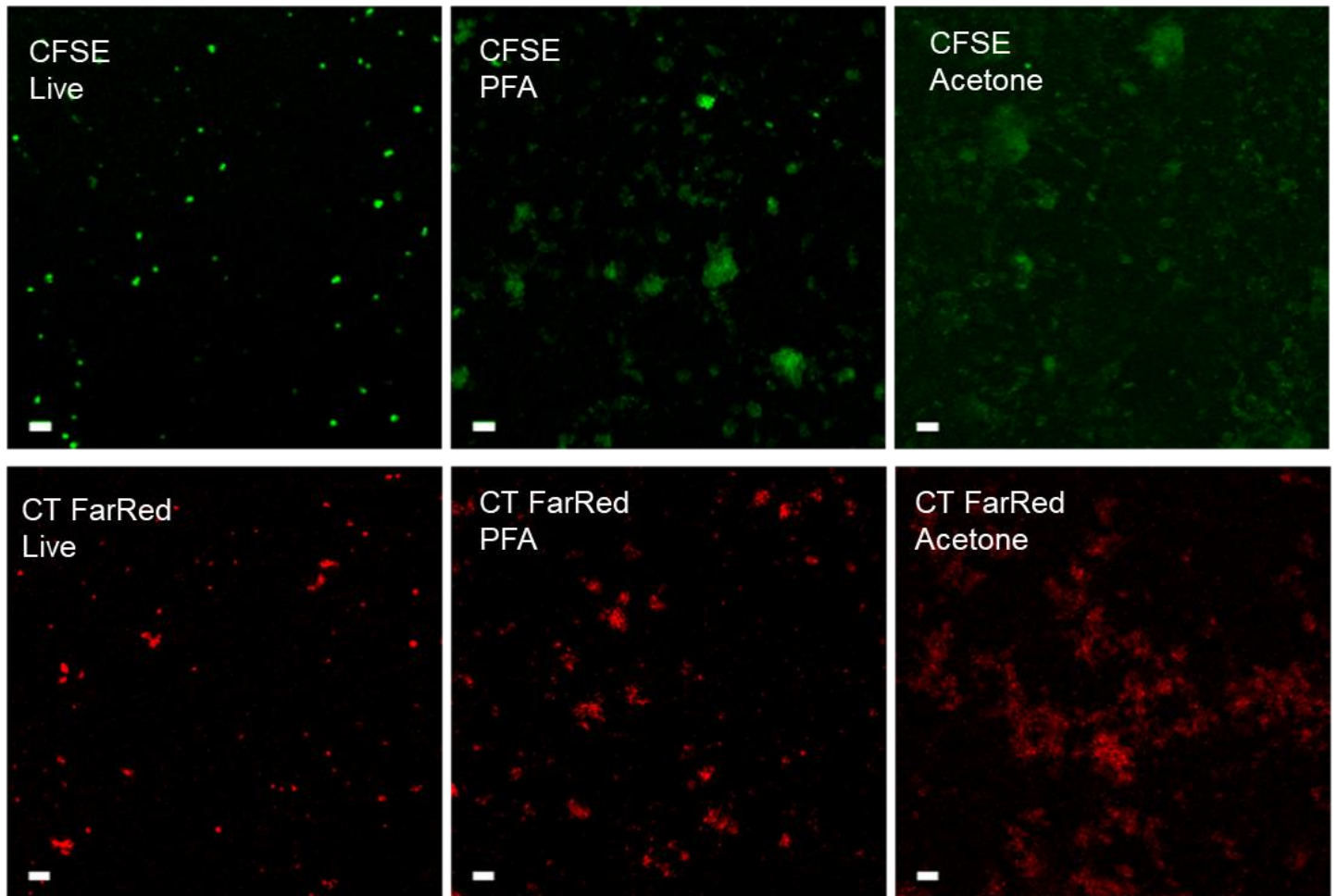


Figure 10. The effect of fixation on CFSE *Orientia*.

Purified *Orientia* were labelled with CFSE or CellTrace FarRed and imaged live or fixed with paraformaldehyde (PFA) or acetone.

Scale bar = 5µm

Bacterial growth was measured when *Orientia* was labelled with CFSE and CellTrace FarRed over seven days using qPCR. There was no difference in bacterial growth after 7 days when labelled with either dye compared with mock treated bacteria in DMSO, as shown by one-way ANOVA statistical test (**Figure 11**). Both dyes can be used reliably to follow the infection cycle without an effect on bacterial growth.

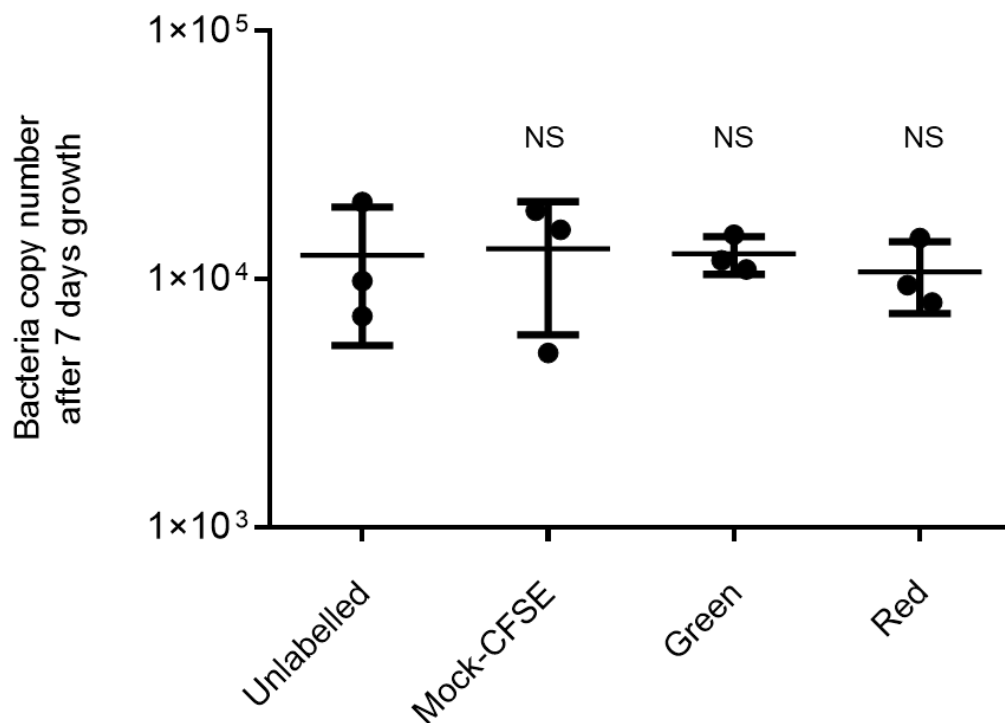


Figure 11. Growth of CFSE labelled *Orientia*

Graph showing the bacterial copy number of one well from a 24 well plate after 7 days growth with or without CFSE green or CT FarRed.. Inoculum used was $1.36 \times 10^4 \pm 1.9 \times 10^3$ bacteria per well (mean \pm SD). Individual replicate values are plotted and the mean \pm SD is shown. The results of the one-way ANOVA statistical test using the no label condition as a comparison are shown.

Work done by Suparat Giengkam.

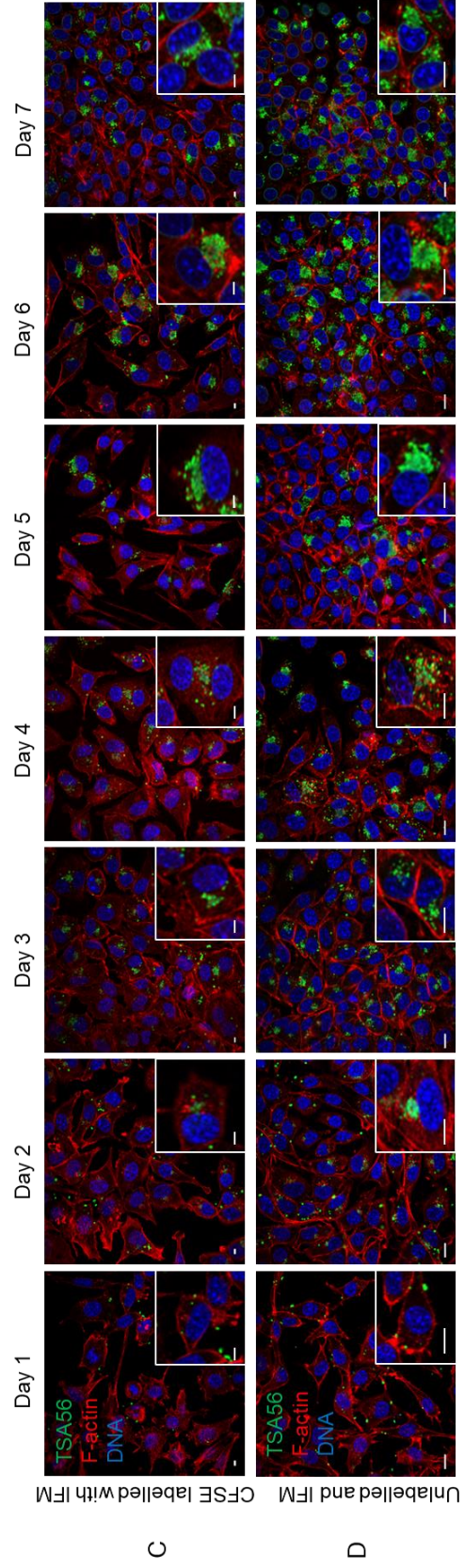
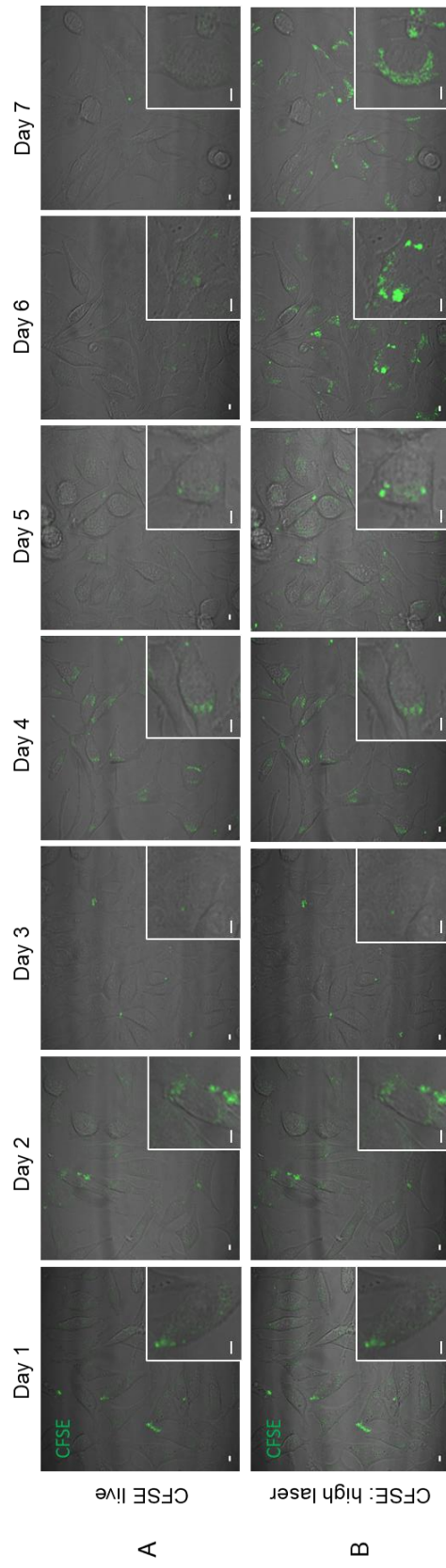
CFSE has commonly been used to follow mammalian cell proliferation by measuring fluorescence intensity over time using flow cytometry [216]. The stable dye becomes diluted once cell division occurs and the decreasing intensity, the division number can be estimated over time [217]. A similar approach was used here to estimate division time in *Orientia*. Bacteria were labelled with CFSE and infected into mammalian cells grown on cover slip slides. For 7 days, bacteria were imaged inside host cells (**Figure 12, A-B**). One chamber slide was used for each time point taken to avoid photobleaching or cell death during microscopy. During each time point a slide was imaged live using confocal microscopy (Zeiss LSM 510) to capture CFSE fluorescence inside host cells. Whilst the FITC laser strength was kept constant for each time point, it was also increased to detect the presence of replicated bacteria faintly labelled with CFSE. To be sure that CFSE had not influenced the infection cycle, slides were fixed and prepared for immunofluorescence microscopy. These images were compared in parallel to infected slides with non-CFSE but antibody labelled *Orientia* (**Figure 12, C-D**).

By day 4, CFSE labelled bacteria were no longer visible by live imaging unless the FITC laser was increased up to power 5. *Orientia* presented the same infection cycle when labelled with CFSE compared with unlabelled bacteria as clarified by antibody labelling. *Orientia* were able to move to the nucleus and remain in the perinuclear region after day 1, until division was completed and exit the host cell from day 4. Therefore, CFSE does not affect the behavior or the infection cycle of intracellular *Orientia*.

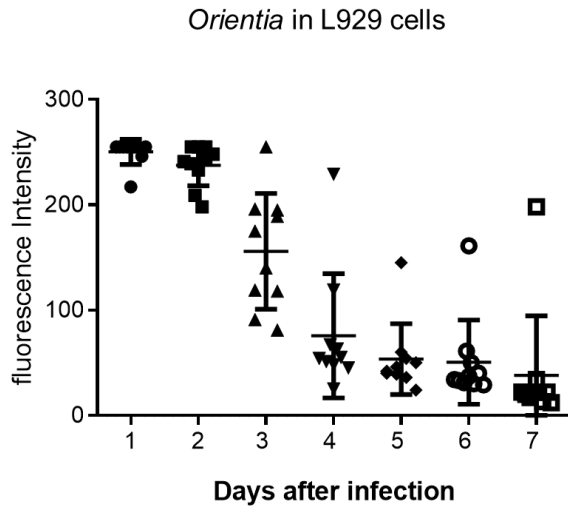
To estimate bacterial division after dilution with CFSE, ten images were taken from live imaging at each time point for quantification. Within these images, intracellular bacteria were chosen at random and the brightest pixel in each bacterium was measured for FITC intensity using ImageJ, a Java based image software program from the US National Institute of Health website (<http://imagej.nih.gov/ij/>). These measurements were compared with bacteria also labelled with

CFSE but not infected into host cells, therefore unable to replicate (**Figure 12, E-F**). These bacteria showed no sign of CFSE dilution unlike the bacteria image inside host cells. The decrease in dilution of CFSE labelled bacteria correlates with the known division time of *Orientia* as shown by our research group [138]. Whilst qPCR measures the genome number, the measurement of CFSE intensity over time can provide a better understanding of bacterial replication.

Bacteria which remain bright after division time has passed may be metabolically inactive or dead. CFSE labelled bacteria which remained stationary and bright may be persisting inside host cells. This trait is common in other bacteria such as *E. coli* [218], *Chlamydia* [219] and *Salmonella* [220] to avoid host immune responses and antibiotic exposure, but is not yet known to exist in *Orientia* intracellular populations.



E



F

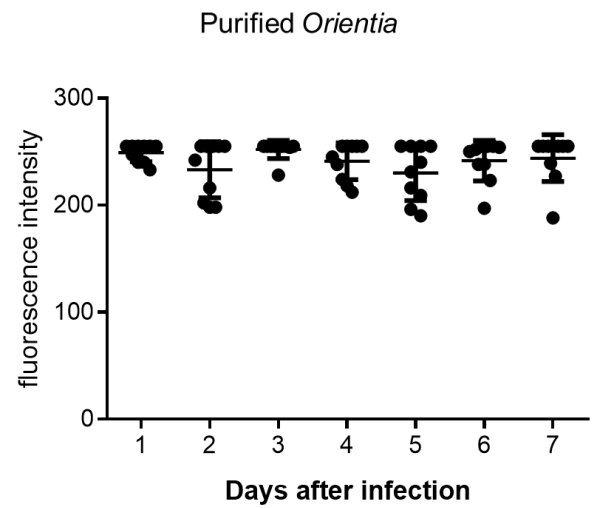


Figure 12. Infection time-course over 7 days showing CFSE labelled bacteria and non-labelled bacteria

A: Purified *Orientia* were labelled with CFSE and imaged live over 7 days at equivalent imaging conditions including laser strength and image acquisition time.

B: This row shows images taken at higher laser strength at the same time period.

C: These samples were CFSE labelled but then fixed with formaldehyde at each time point and labelled with TSA56.

D: *Orientia* were not labelled with CFSE in this sample but only labelled with TSA56.

E: Pixel intensity of intracellular bacteria labelled with CFSE over 7 days.

F: Pixel intensity of bacteria in media labelled with CFSE over 7 days.

Scale bar = 5µm

3.3.c Syto9

Syto9 (S34854, BacLight™, Molecular Probes, USA) is a cell permeable fluorescent nucleic acid stain which can label DNA and RNA live in eukaryotic and bacterial cells. This stain is helpful in determining live cells whilst companion component propidium iodide (PI) labels dead cells. Syto9 (excitation 485, emission 498) enters live and dead cells whilst PI (excitation 533, emission 617) will only enter cells with disrupted membranes. When both dyes are present, PI will present a stronger affinity for nucleic acid, displacing syto9.

Syto9 at 5nM was used to detect *Orientia* by microscopy at various stages of the infection cycle. This dye was useful in detecting bacteria during live imaging at specific time points, as the dye can easily be added to culture media. Host nuclei will be also labelled but bacteria were obvious in infected cells compared to uninfected host cells. Syto9 can be used short term during live imaging to locate intracellular bacteria. In host cells infected with *Orientia* and labelled with syto9 at day 4 of the infection cycle, bacteria were noted to be moving energetically in the perinuclear area. Syto9 cannot be fixed and is toxic to cells, affecting bacterial growth at long durations of labelling [221].

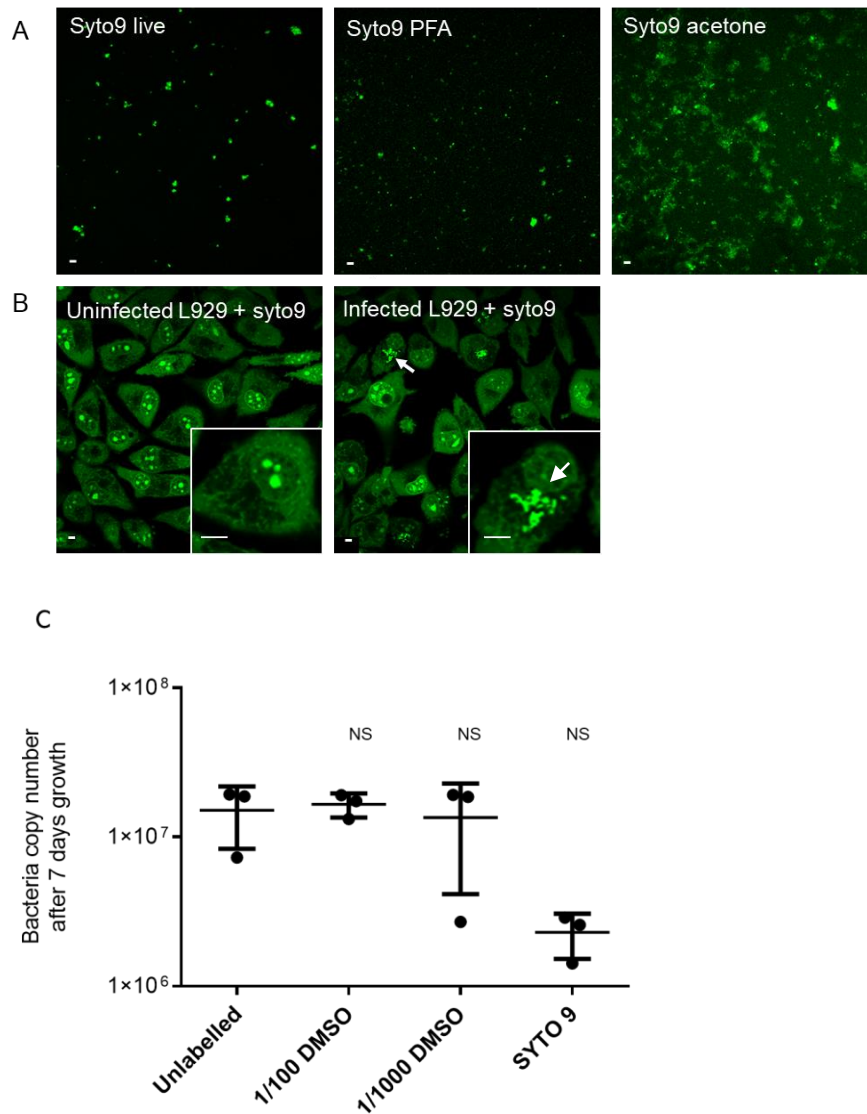


Figure 13. Purified *Orientia* labelled with DNA binding label SYTO9

A: Live and fixed with acetone and PFA, and inside host cells

B: L929 cells were infected with *Orientia* and imaged after the addition of SYTO9. The white arrows indicate the presence of intracellular bacteria.

C: Graph showing the bacterial copy number from one well of a 24 well plate with or without DMSO or SYTO9. One-way ANOVA test was carried out to compare with the no label condition. Work done by Suparat Giengkam.

Scale bar = 5µm

3.3.d HADA

Fluorescent dyes or probes may sometimes label unspecifically leading to high background labelling or false positives. To avoid this problem, probes which target specific bacterial structures have been produced. Specifically, probes have been developed which target the unique structure of peptidoglycan in bacteria. It was previously reported that *Orientia* lacks peptidoglycan [222] but recent results have led to this being disputed, which will be discussed later.

During peptidoglycan biosynthesis, a D-alanine dipeptide is inserted into the peptide chain by enzyme MurF. Fluorescent probe, produced by M. VanNieuwenhze, HCC amino D-alanine (HADA) can be inserted into sites of new synthesis and is conjugated with a fluorophore (emission maximum, 450 nm blue) [223, 224]. This incorporation has already been shown for *Chlamydia*, *B. subtilis* and *E. coli* [224] [225]. HADA was used to label *Orientia* in fixed and live samples. When used at 1mM, *Orientia* labelled as rings, indicating HADA successfully entered a cell wall structure. HADA was also shown to have no effect on the growth of bacteria when compared with non-HADA labelled bacteria. HADA can be imaged live and is fixable with paraformaldehyde and acetone but generates a high background causing imaging to become difficult.

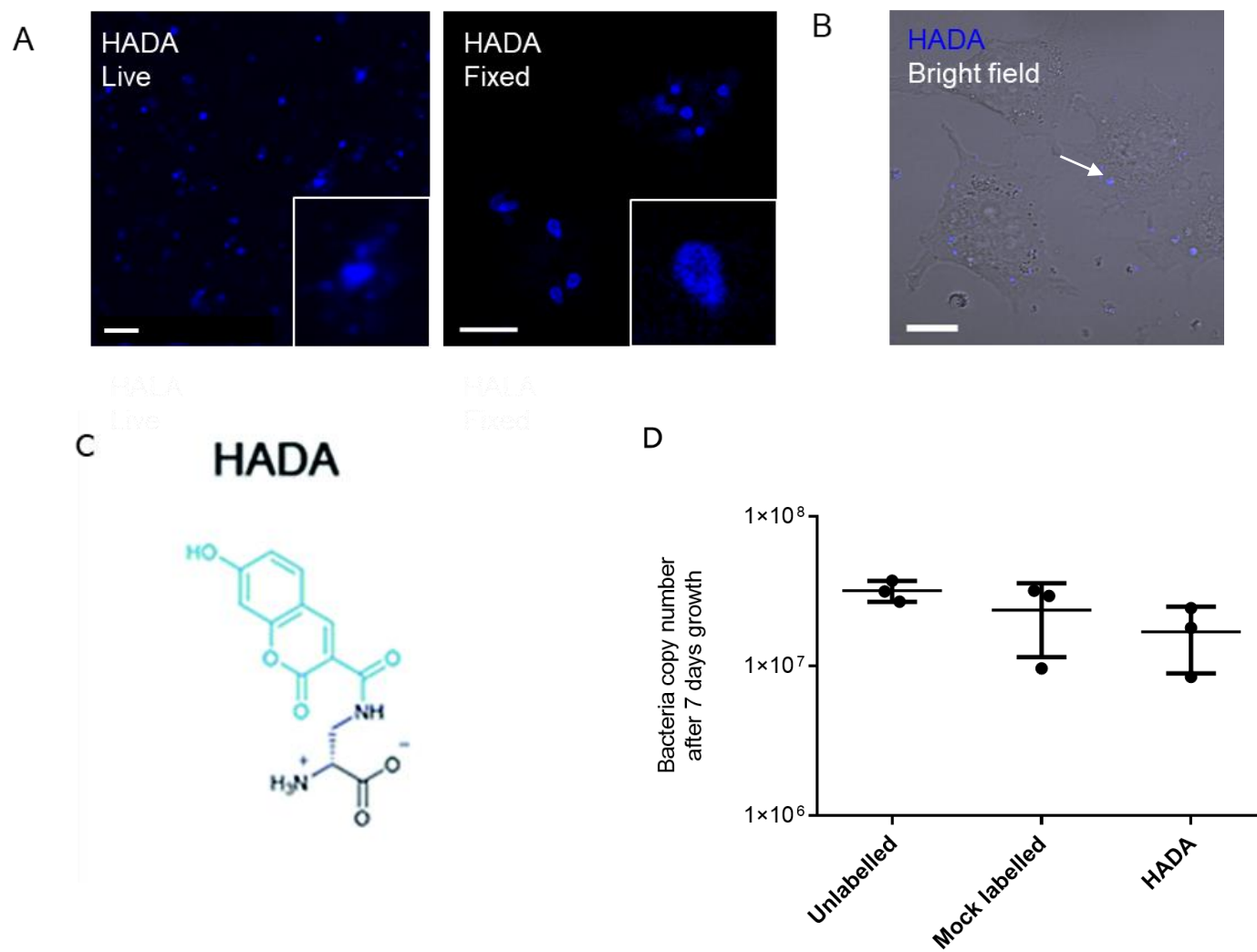


Figure 14. Purified *Orientia* labelled with peptidoglycan label HADA

A: Live and fixed HADA labelled *Orientia* with PFA

B: L929 cells were infected with *Orientia* labelled with HADA and imaged immediately after entry

C: The molecular structure of fluorescent D-amino acid HADA with the fluorophore moiety highlighted.

D: Graph showing the bacterial copy number in one well of an infected 24 well plate in the presence or absence of HADA. One-way ANOVA statistical test was carried out to compare to the no label condition. Work done by Suparat Giengkam.

Scale bar = 5µm

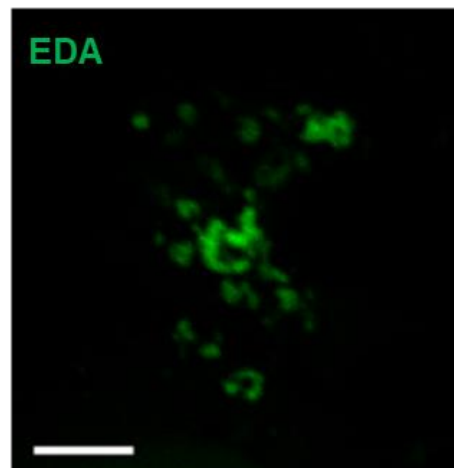
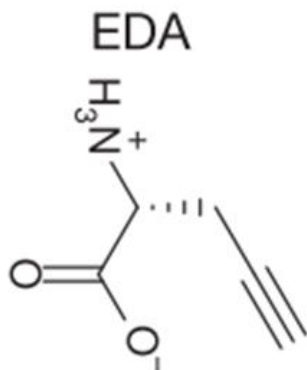
3.3.e EDA and EDA-DA

Using a probe such as HADA gives the advantage of specific bacterial labelling. However the incorporation of HADA may not be as efficient because the fluorescent moiety attached to the synthetic D-amino acid is quite large (**Figure 14, C**). An alternative and potentially less perturbative label to HADA are clickable probes, Ethynyl-D-alanine (EDA) and Ethynyl-D-alanyl-D-alanine (EDA-DA), produced by our collaborator M. VanNieuwenhze [223]. These probes are 'clickable' alkyne modified D-alanine D-alanine dipeptide probes which label peptidoglycan. The labelling method of these probes involves a click chemistry reaction with a fluorophore conjugated to an azide tag, to be visualised after fixation. Instead of a fluorophore, an alkyne tag is attached to the probe so allow for better incorporation.

Both probes were tested with *Orientia* for specificity (**Figure 15**). At 1mM both EDA and EDA-DA labelled the entire cell and sometimes, like HADA labelling, presented ring-like structures. For simplicity, only EDA was used in future experiments due to the similar results generated from both probes which led to the conclusion that both are incorporated into the peptidoglycan structure in the same way.

These results indicate EDA and EDA-DA were incorporated into the peptidoglycan peptide structure of *Orientia*, giving evidence that *Orientia* does in fact have a peptidoglycan-like structure. In the case of pathogenic strains *Chlamydia*, which also possess only minimal peptidoglycan, peptidoglycan labelling was only seen at the septum [225]. Septum labelling was never observed in *Orientia*.

A



B

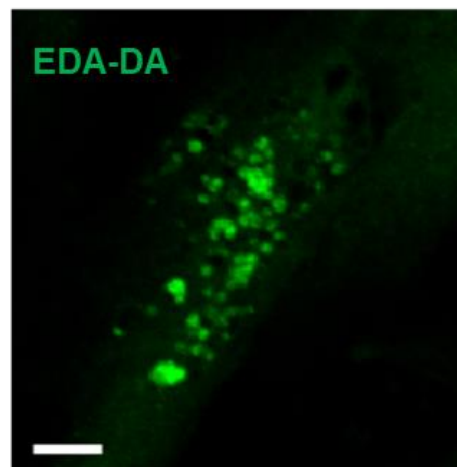
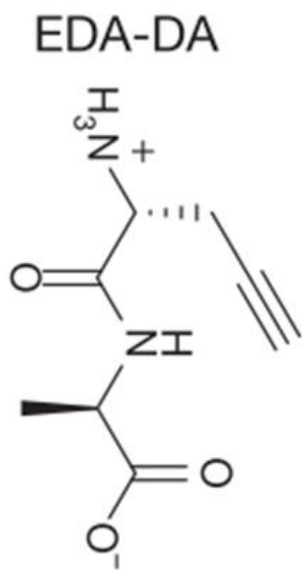


Figure 15. Intracellular *Orientia* labelled with peptidoglycan probes Ethynyl-D-alanine (EDA) and Ethynyl-D-alanyl-D-alanine (EDA-DA)

A: The molecular structure of EDA and EDA labelled *Orientia* inside PTK2 cells day 3 post infection.

B: The molecular structure of EDA-DA and EDA-DA labelled *Orientia* inside PTK2 cells day 3 post infection.

Scale bar = 5µm

3.3.f Vybrant dyes

Membrane dyes are commonly used to label bacteria [226]. Lipophilic dyes DID, DIO and DIL (Molecular Probes, USA) can be added directly to cell culture to label lipid membranes and fluoresce at a range of colours. Bacteria can be labelled separately from host cells so that there is no possibility of diffusion of the label to host cells, decreasing the possibility of non-specific labelling. Labelling *Orientia* with DID, DIO and DIL displayed membrane and cytoplasmic labelling (**Figure 16**). Bacteria were seen clearly entering and inside the host cells during infection. However, the dyes were unable to be fixed with paraformaldehyde or acetone. Additionally, the labelling protocol of these vybrant dyes, recommends use of a specialised buffer, opti-mem (31985070, ThermoFisher, USA), which affected the growth of *Orientia*. Labelling may be optimised in other buffers, less toxic to the bacteria but these were not tested at this time. As the dye is incorporated into the membrane, it may be useful for studying outer membrane vesicles which may bud from bacteria during infection. These dyes are best used for short term labelling.

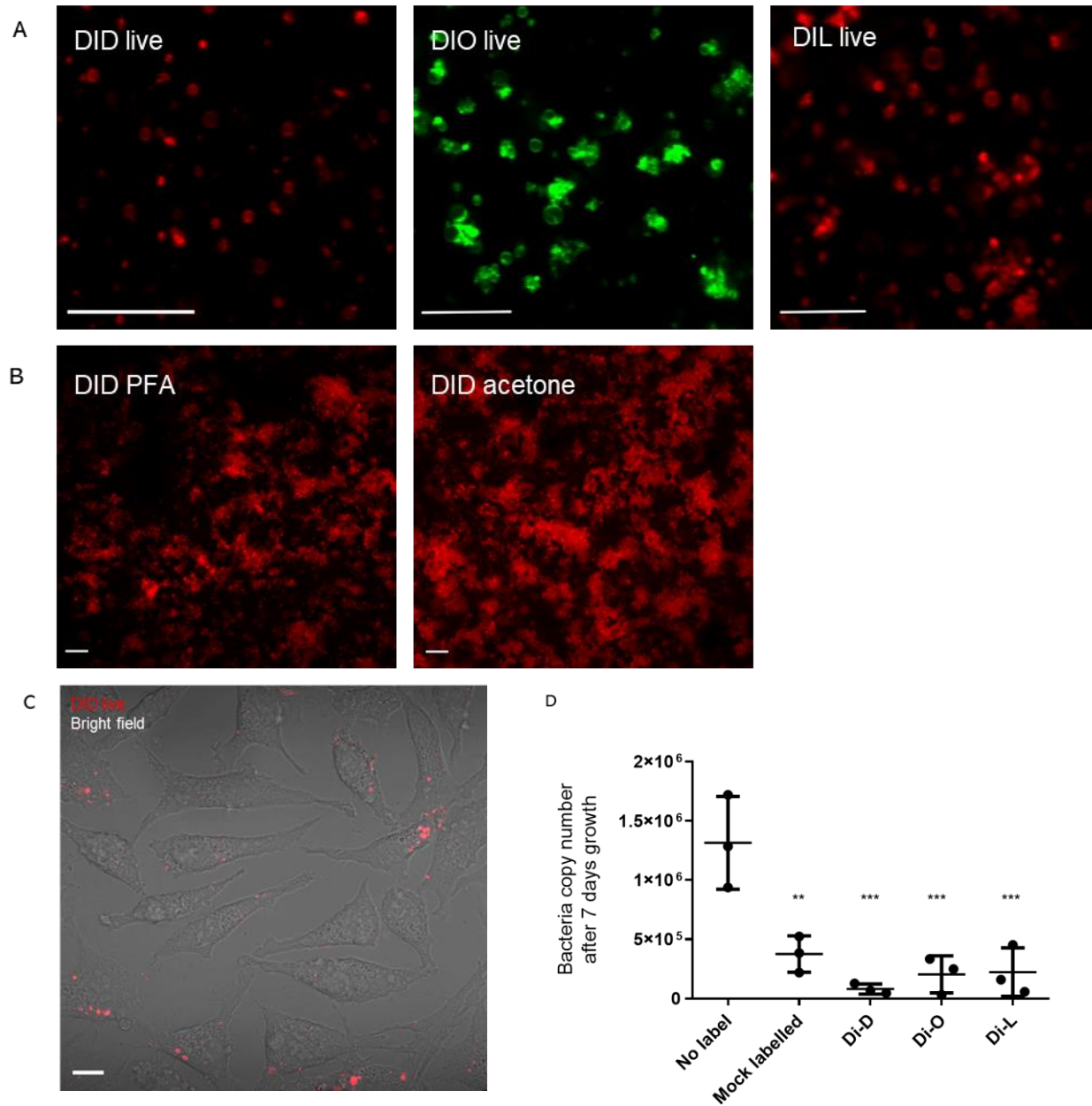


Figure 16. Purified *Orientia* labelled with vibrant membrane labels DID, DIO and DIL

A: *Orientia* labelled live with each vibrant dye.

B: *Orientia* labelled live with DID and fixed with PFA and acetone.

C: L929 cells infected with *Orientia* labelled with DID image live by confocal microscopy.

D: Graph showing the bacterial copy number of one well from an infected 24 well plate. One-way ANOVA statistical test was carried out to compare to the no label condition. ** $P \leq 0.01$; *** $P \leq 0.001$.

Scale bar = 5 μ m

3.3.g Clickable methionine

Clickable chemical probes can be used to study the incorporation and localization of specific molecules. One such probe is the methionine derivative, L-Homopropargylglycine (ThermoFisher, USA) or L-Azidohomoalanine (Click chemistry tools, USA). These are 'clickable' probes that provide a non-toxic and easy labelling of methionine for protein synthesis detection for fixed imaging. These analogs consist of either an alkyne or azide moiety conjugated to methionine which are taken up by bacteria during protein synthesis and are visualised in fixed cells after a 'click' reaction with a fluorophore. This technique is commonly used to label host and bacterial protein synthesis [227]. These probes were used to label metabolically active *Orientia*. Intracellular bacteria were labelled with 5uM probe for 30 minutes before fixation. This time was enough to label *Orientia* and bacteria labelling was presented as cytoplasmic (**Figure 17**). This technique is advantageous for labelling *Orientia* as metabolically active bacteria can be distinguished at various stages of the intracellular life cycle.

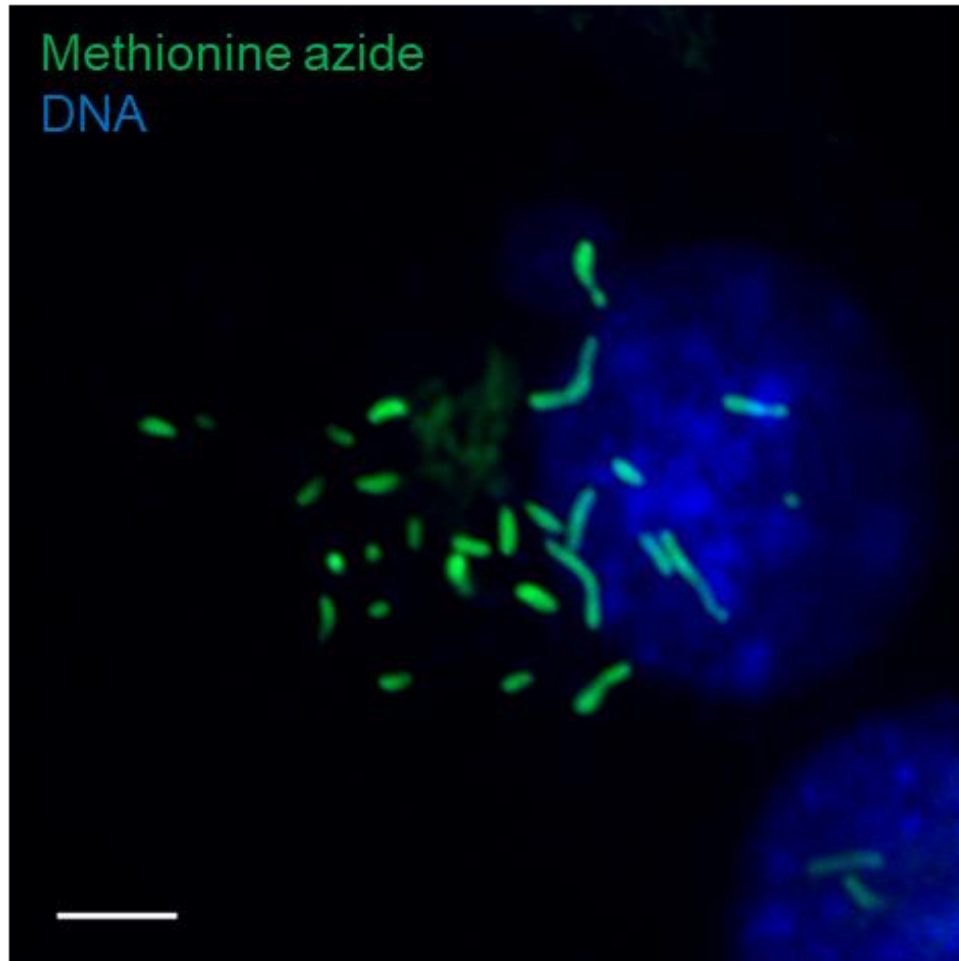


Figure 17. Chemical probe methionine azide or alkyne can label metabolically active *Orientia*

Intracellular *Orientia* labelled with HPG methionine probe inside L929 cells, 3 days post infection. The methionine binding probe was added 30 minutes prior to fixation.

Scale bar = 5µm

3.3.h RNA FISH

Immunofluorescence microscopy is used to study expression levels and patterns of proteins inside cells. It would be desirable to have a strategy to measure expression levels and patterns of RNA transcripts inside cells. This would enable comparison of transcription and translation levels and allow the study of genes where antibodies were not available. This was done by using single cell RNA fluorescence in-situ hybridisation (RNA FISH). In this, an oligonucleotide is synthesized that is complementary to the gene of interest, and this is conjugated to a fluorophore. Cells are fixed and permeabilized, and an overnight hybridisation step allows the probe to bind to its target. RNA FISH targeting 16S RNA at 1ng/ul was developed in the lab by Suparat Giengham and then I used it to successfully label intracellular *Orientia* (**Figure 18**). 16S RNA is expressed in ribosomes and since ribosomes will always be present at some level it was assumed that all bacteria should be labelled with this stain and this could be used as a control to counterstain all bacteria. Using RNA FISH against other genes will allow the study of differences in gene expression within a population and at different times after infection.

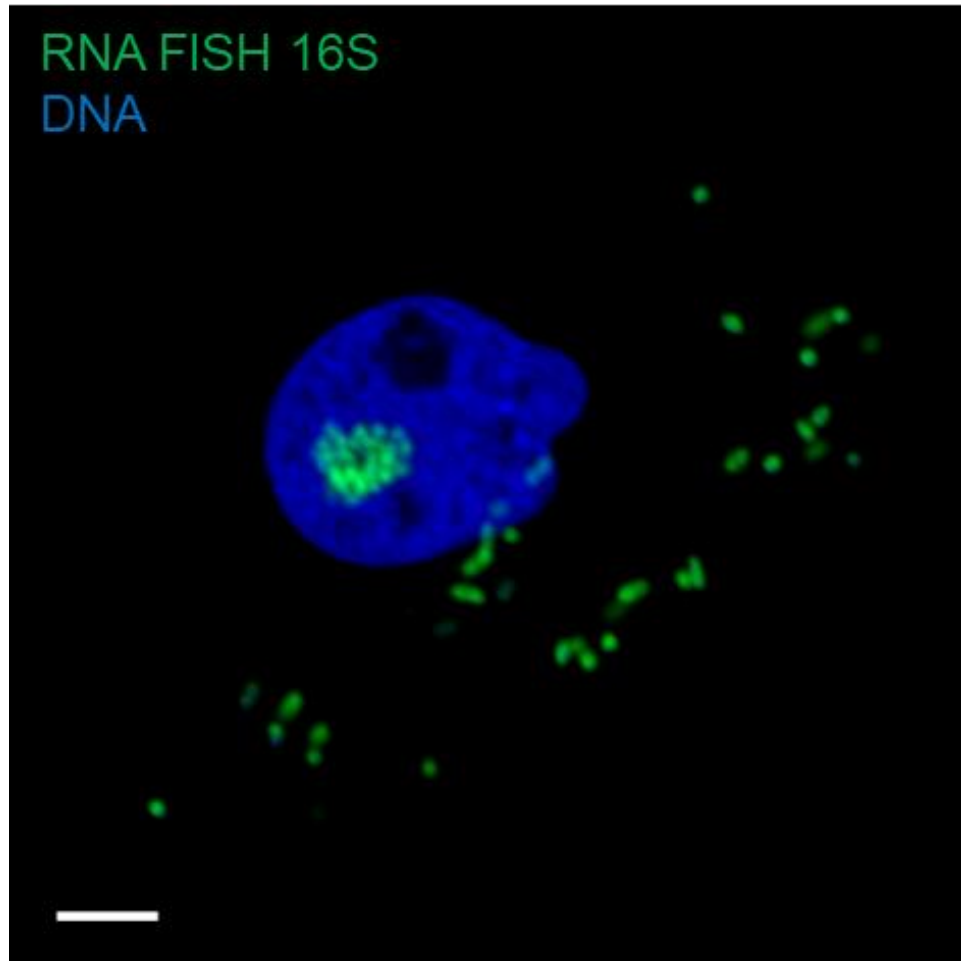


Figure 18. RNA FISH labelling of *Orientia*

Intracellular *Orientia* labelled by RNA fluorescence in situ hybridization (FISH) inside PTK2 cells. Bacteria were fixed with PFA at day 3 and labelled with RNA FISH for 16s.

Scale bar = 5µm

3.4 Discussion

Fluorescent imaging is a vital tool for tracking the infection cycle of an obligate intracellular bacterium. Without genetic manipulation, *Orientia* must be labelled by an alternative mechanism. However, the optical and physical properties of the label can affect the function of the bacteria. A range of labels were tested for *Orientia* which successfully labelled the bacterial cytoplasm, the cell wall, nucleic acids or the cell membrane.

First, antibodies were generated against major surface proteins of *Orientia*. The technique of immunofluorescence labeling is relatively easy and fast to carry out. As cells are already preserved by fixation, there is no danger of an effect of the antibody to the bacteria. The only concern would be the photostability of the fluorophore used as a secondary antibody, but this was not the case as all antibodies were stable and long-lasting for imaging. The monoclonal antibodies proved to be highly specific to the antigen it recognised as shown by Western blot and microscopy images. The polyclonal antibodies did show some unspecific binding so the protocol for these antibodies may require the addition of a blocking buffer such as bovine serum albumin (BSA) to reduce non-specific antibody binding. Each antibody labelled at various times of the infection cycle which is useful for understanding the role of surface proteins interactions in the host cell. The labelling morphology was also interesting as some surface proteins labelled throughout the membrane surface (TSA56, TSA47) whilst others (ScaC and TSA22) appeared to be 'spotty'. This indicates the abundance of the surface protein on the membrane at a given time in the infection cycle and may help to understand how *Orientia* uses its surface proteins to interact with host proteins.

CFSE and CellTrace FarRed dyes successfully labelled the cytoplasm of *Orientia*. The technique of labelling with the dyes is extremely easy and fast as isolated bacteria are labelled before infection into host cells. Only live imaging can be carried out as fixation after dye integration presented change in fluorescence intensity and bleeding of the dye into the external environment.

The CFSE dyes proved to be photostable and long lasting in *Orientia* labelled with CFSE. Growth or behaviour of bacteria was not affected so long-term imaging can be carried out if needed. Although CFSE dyes are long lasting, they also dilute over time as the bacteria divide. But this was a good and alternate technique to estimate the division of bacteria over time by live imaging instead of measuring the copy number of a gene by qPCR. Furthermore, CFSE labelled bacteria which remained bright after division indicate that not all bacteria divide inside the host cells. These may be dead or captured bacteria about to be disposed but the trait of bacterial persistence could also be an option. This has never been shown to exist in *Orientia* populations but could explain cases of persistence in mammals [228]. CFSE dyes also presented specificity to the bacterial cytoplasm and did not leach out into host cells.

SYTO9 successfully labelled DNA in *Orientia*. Labelling of SYTO9 was easy and quick as the label is directly added to infected cells. Whilst SYTO9 was useful in imaging live bacterial movements, the dye cannot be used for fixed imaging as the fluorescence leached out of bacterial cells. SYTO9 was not stable and photobleached soon after live imaging was performed. The dye also affected the growth of *Orientia* so cannot be used for long-term imaging. SYTO9 labelled DNA in *Orientia* but also labelled host DNA. This could potentially complicate image analysis although bacteria were easy to identify in infected cells. This problem does not arise when imaging bacteria isolated from host cells.

Vybrant lipophilic dyes, DIO, DIL and DID also successfully labelled *Orientia* but only for live imaging. Fixed labelling of the dyes did not image well. The technique is easy as isolated bacteria can be labelled and added to host cells for imaging. However, the vybrant dyes affected the growth of *Orientia* and cannot be used for long-term imaging due to the lack of photostability. Also, after bacteria were added to host cells post labelling, the dye was seen around the host cell. This may have been residual dye or membrane fragments from *Orientia*. If the latter, it would be a useful technique to follow the blebbing of outer membrane vesicles during the infection cycle

[229] or to follow bacterial cell division. Although the dye was specific to the lipid membrane in most cases, it was also localised in the cytoplasm in some bacteria. Bacteria must be labelled in isolation from host cells otherwise the dye will also label the lipid membrane of the host cells.

Fluorescent probes specific to bacterial peptidoglycan were also tested. HCC amino D-alanine (HADA) probe was able to label the cell wall of *Orientia* for fixed and live imaging. The technique of labelling was simple but did take a longer duration to be incorporated into the cell wall. During live imaging, the probe was photostable over time and did not affect the growth or behaviour of *Orientia*. Cell wall labelling was clear in fixed and live images. Although the probe appeared to label specifically, a high background after fixation was generated, especially inside host cells. Also, the probe quickly became bleached during fixed imaging. This would make peptidoglycan labelling during the intracellular infection cycle difficult.

Other peptidoglycan specific, clickable probes, Ethynyl-D-alanine (EDA) and Ethynyl-D-alanyl-D-alanine (EDA-DA) were used to achieve more specific labelling. These probes do not consist of a large attached fluorophore as HADA does. The technique for EDA and EDA-DA labelling is slightly more complicated due to the click reaction needed for fluorescence. Only fixed imaging can be carried out and the probes must be added to infected cells for a duration of time. Following fixation, a click chemistry reaction is performed along with antibody labelling. This allows a counter label for the peptidoglycan labelling to show specificity of the probe. EDA and EDA-DA both were photostable and did not bleach easily. Labelling of the probes varied from experiment to experiment which indicates there must be other factors affecting labelling or bacterial peptidoglycan synthesis is only occurring at certain points of the infection cycle. Probe labelling did not vary between EDA and EDA-DA. Labelling was mostly visualised uniformly on the whole body of the bacteria and in a small population, the cell wall was labelled. This difference in labelling can further be explored by understanding when and how peptidoglycan synthesis occurs in *Orientia*.

Another option used for labelling was methionine specific probes. These probes are integrated as methionine is incorporated during bacterial protein synthesis. The technique for these probes is very easy as just a short incubation time with infected cells is needed for optimal results and there is no effect on bacterial growth or behaviour. The probe can only be visualised after fixation and click chemistry labelling. The probes were stable and did not bleach after imaging. Probes also presented specificity, as labelling was cytoplasmic. However, methionine probes will also label the host cell, since the probe does not discriminate between host or bacterial protein synthesis. This did not prove to be a problem since the bacteria labelled so intensely. This technique is useful for labelling only metabolically active bacteria. This would allow visualisation of when protein synthesis begins once bacteria enter host cells, which relates to when the bacteria start division or cell wall synthesis. Counter-labelling with an antibody can be performed to compare active bacteria vs non active bacteria.

Lastly, RNA FISH was performed, which was designed by Suparat Giengham to bind with a sequence from the 16s gene. This protocol for RNA FISH is quite lengthy and requires many stages for optimal imaging. As it is added after fixation, there is no effect on bacterial growth or behaviour. Labelling is specific and bacteria labelling appears to be 'spotty' inside the cell, which may indicate mRNA and ribosome locations.

In this research, formaldehyde fixation was commonly used to preserve samples. Images may have been improved by using other fixatives such as organic solvents, which may be better to preserve nucleic acid content and decrease high backgrounds after immunostaining. Alternate fixative techniques will be explored for future work.

Finding the appropriate label for *Orientia* was time consuming but valuable for future research. Though all are useful, each label is beneficial for a specific research question and at a specific time of the bacterial infection cycle.

One long standing question is the topic of peptidoglycan in *Orientia*. The success of the D-alanine probe uptake into the cell wall of *Orientia* may help answer whether the bacteria can synthesise peptidoglycan. This will be presented in the next chapter.

Label	Fixed	Live	Advantages	Disadvantages
Antibodies	✓		Specific to bacteria proteins, no host labelling	Expensive to produce
CFSE/CellTrace FarRed		✓	Can be used to estimate division	Dilutes over time
SYTO9		✓	Labels only live bacteria	Toxic, affects bacterial growth Labels host nuclei
HADA	✓		Incorporated into the cell wall	Does not label throughout infection cycle
EDA-DA	✓		Incorporated into the cell wall	Does not label throughout infection cycle
Vybrant		✓	Can be used to study outer membrane vesicle budding	Toxic, affects bacterial growth
Clickable methionine	✓		Labels only metabolically active bacteria	Does not label all bacterial population
RNA FISH	✓		Can follow gene expression over infection cycle	Low throughput and complicated protocol

Table 5. A summary of labels tested for *Orientia* in this thesis

Chapter 4

Peptidoglycan in *Orientia tsutsugamushi*

Most of this research has been published: Evidence for a peptidoglycan-like structure in *Orientia tsutsugamushi*, Atwal S, Giengkam S, Chaemchuen S, Dorling J, Kosaisawe N, VanNieuwenhze M, Sampattavanich S, Schumann P, Salje J. 2017. Mol. Microbiol

4.1 Introduction

It was previously thought that *Orientia* did not synthesise peptidoglycan as major constituents of the structure were not found by chemical analysis. Also its insensitivity to β -lactams indicated a lack of peptidoglycan structure [222] [230] [231]. However, *Orientia* has a nearly complete set of genes for peptidoglycan synthesis [25] [1] (**Figure 19**) which makes the existence of some sort of peptidoglycan-like structure in its cell wall very likely. Furthermore, *Orientia* is recognised by host immune response Nod1 [201]. But still little is known about the structure of peptidoglycan in *Orientia*. Also, although Gram-negative, *Orientia* does not have LPS, as shown by the lack of detection of lipopolysaccharide from SDS-PAGE analysis [222] and the lack of any genes for LPS biosynthesis [1].

The obligate intracellular bacterium *Chlamydia* was also thought to not synthesise peptidoglycan even though it is susceptible to drugs which target peptidoglycan and can produce PBPs, as well as having most of the genes needed to produce peptidoglycan. The inability to detect peptidoglycan became known as the *Chlamydia* paradox [232]. However, by the use of mass spectrometry and fluorescent probes integrated into the peptide structure, peptidoglycan has now been shown to exist in *Chlamydia* [223, 233]. Peptidoglycan was found to exist in the division septum at very low amounts. As *Chlamydia* do not have FtsZ, which coordinates peptidoglycan

bacterial division at the septum, other cytoskeleton components such as MreB and RodZ are involved in the organisation of division [234]. Peptidoglycan in *Chlamydia* may instead take the role of FtsZ and exist solely as a lipid II precursor [193] not as a distinct sacculus. Without a classic peptidoglycan sacculus in its cell wall, *Chlamydia* species may instead maintain osmotic stability from disulfide bonded outer membrane proteins instead during their infection cycle [235] [236].

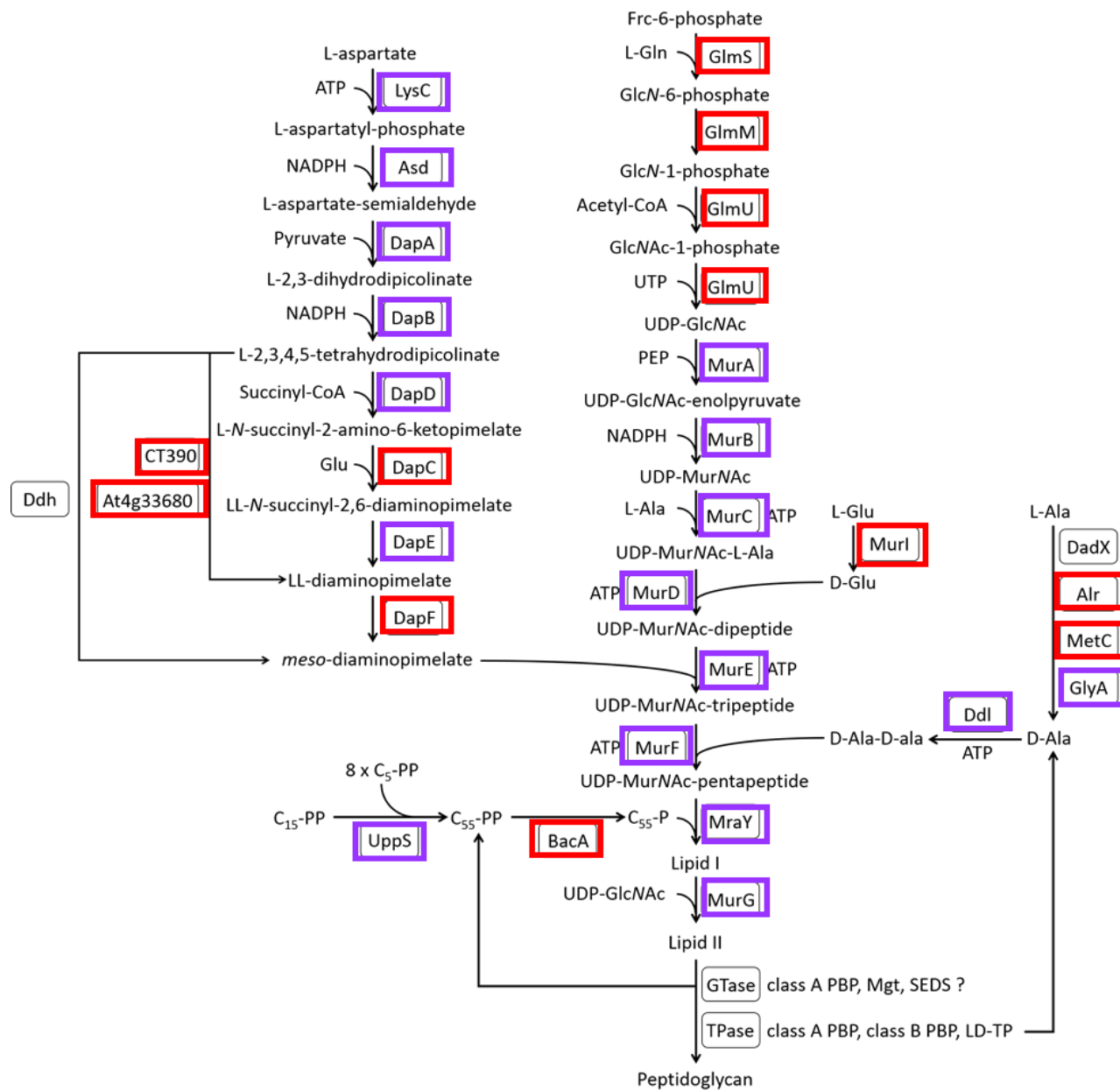


Figure 19. The peptidoglycan synthesis pathway

The proteins involved in peptidoglycan synthesis for Gram-negative bacteria. Purple boxes indicate genes which are found in the genome of *Orientia*. Red boxes indicate genes which are absent in the genome of *Orientia*.

4.2 Peptidoglycan in *Orientia*: Objectives

Objective 1: To detect peptidoglycan-specific amino acids in *Orientia*

- Approach: To use mass spectrometry analysis to assess the presence or absence of *meso*DAP in isolated *Orientia* bacteria
- Expected outcome: If present, *meso*DAP in isolated bacteria will be identified based on its chromatographic peak compared to the standard *meso*DAP control

Objective 2: To measure the expression of genes involved in peptidoglycan synthesis in *Orientia*

- Approach: To measure the gene expression of specific genes (*murA*, *murD*, *murF*, *ddl* and *pbpA2*) using qRT-PCR
- Expected outcome: If peptidoglycan biosynthesis is occurring it is expected that genes involved in this pathway will be expressed

Objective 3: To test the sensitivity of *Orientia* to cell wall targeting drugs

- Approach: To measure the sensitivity of cell wall targeting drugs (phosphomycin, penicillin, d-cycloserine, meropenem, imipenem, mecillinam and tazobactam) on the growth of *Orientia* by qPCR and to analyse morphology of bacteria by immunofluorescence microscopy
- Expected outcome: The viability and morphology of *Orientia* will be affected if peptidoglycan is present in the cell wall

Objective 4: To determine whether peptidoglycan in *Orientia* has a polymerised glycan backbone

- Approach: To measure the sensitivity of *Orientia* to lysozyme and mutanolysin by qPCR and fluorescence microscopy
- Expected outcome: If lipid II is polymerised by glycosyltransferase activity, peptidoglycan structure will be affected which would inhibit growth of *Orientia*.

Objective 5: To identify the subcellular localisation of peptidoglycan in *Orientia*

- Approach: To use D-alanine based peptidoglycan-specific chemical probes to label *Orientia*
- Expected outcome: Presence of peptidoglycan would be shown by localisation of probes to the septum or the cell wall. If labelling is disrupted by the addition of a cell wall targeting drug, this would be further proof of the presence of peptidoglycan.

Objective 6: To determine the effect of osmotic stress on *Orientia*

- Approach: To expose isolated *Orientia* cells to conditions of varying osmotic pressure and to determine the effect on bacterial morphology by immunofluorescence microscopy
- Expected outcome: The resistance of isolated bacteria to osmotic stress would indicate the presence of a load bearing structure such as peptidoglycan

Objective 7: To determine if a network of disulphide cross-linked outer membrane proteins confers additional structural protection to *Orientia*

- Approach: To treat *Orientia* with reducing agents and measure the effect on bacterial shape (by immunofluorescence microscopy) and infectivity (by host infection studies).
- Expected outcome: If the addition of reducing agent affects bacterial morphology this would suggest that disulphide cross-linked surface proteins play a role in conferring structural support

4.3 Results

4.3.a MesoDAP (work done by Peter Schumann and Suwittra Chaemchuen)

First, to help answer if *Orientia* has peptidoglycan, detection of a component of this structure was sought. Meso-Diaminopimelic acid, also known as *meso*DAP, is an amino acid only found in bacterial cell walls, unique to the peptide chains of peptidoglycan. Detection of this would be a strong indicator of peptidoglycan synthesis. *meso*DAP has not previously been detected due to the outdated technique of gas chromatography previously used [222].

The identification of *meso*DAP, was achieved by the more sensitive gas chromatography-mass spectrometry (GC/EIMS) analysis in whole bacteria. Although the genome of *Orientia* does not include the genes for the complete pathway of *meso*DAP production, *meso*DAP was detected in purified bacteria. A chromatographic peak was detected matching the peak of *meso*DAP (**Figure 20**). This indicates that *Orientia* is synthesising meso-DAP using an alternate and unknown pathway. Since the data was taken from whole bacteria, purification of the sacculus will provide more reliable and accurate data.

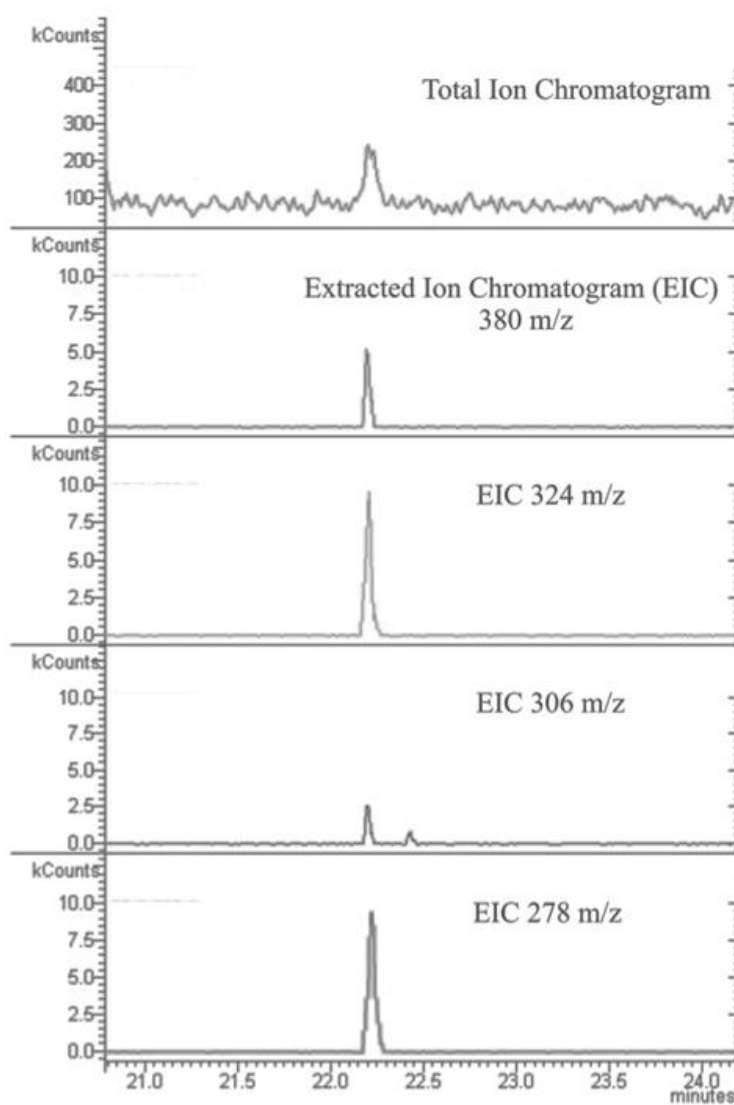


Figure 20. Meso-DAP identification in *Orientia* by mass spectrometry analysis

Hydrolysed bacteria show a peak at 22.2 min and a set of fragment-ions at 380, 324, 306 and 278 m/z.

This work was done Peter Schumann and Suwittra Chaemchuen

4.3.b Gene expression (work done by Suparat Giengkam)

If peptidoglycan synthesis is occurring, then genes involved in its biosynthesis must be switched on. With this reasoning, specific peptidoglycan genes were measured by qPCR over the course of a 7-day infection. These genes were normalised against the house-keeping gene *mipZ*. Genes *murA*, *murD*, *murF*, *ddl* and *pbp2* were all expressed during the 7-day infection (**Figure 21**).

MurA is expressed quite consistently during infection whereas MurD is expressed at much higher levels during the first few days of infection. MurD catalyses the addition of D-glutamic to MurNAc-L-alanine in the cytoplasm, which results in the dipeptide L-Ala-D-Glu. This may suggest that MurD is preparing new peptidoglycan precursors before new incorporation of peptidoglycan, before the bacteria divide. MurF attaches the D-Ala-D-Ala dipeptide to the MurNAc tripeptide, also in the cytoplasm and is expressed highly just before division. Pbp2 also presented this pattern of higher expression levels just before division. This may suggest that *Orientia* is preparing for peptidoglycan synthesis and accumulating lipid II precursors in preparation. Note that these experiments measured the levels of RNA, and these may not reflect differences at the protein level.

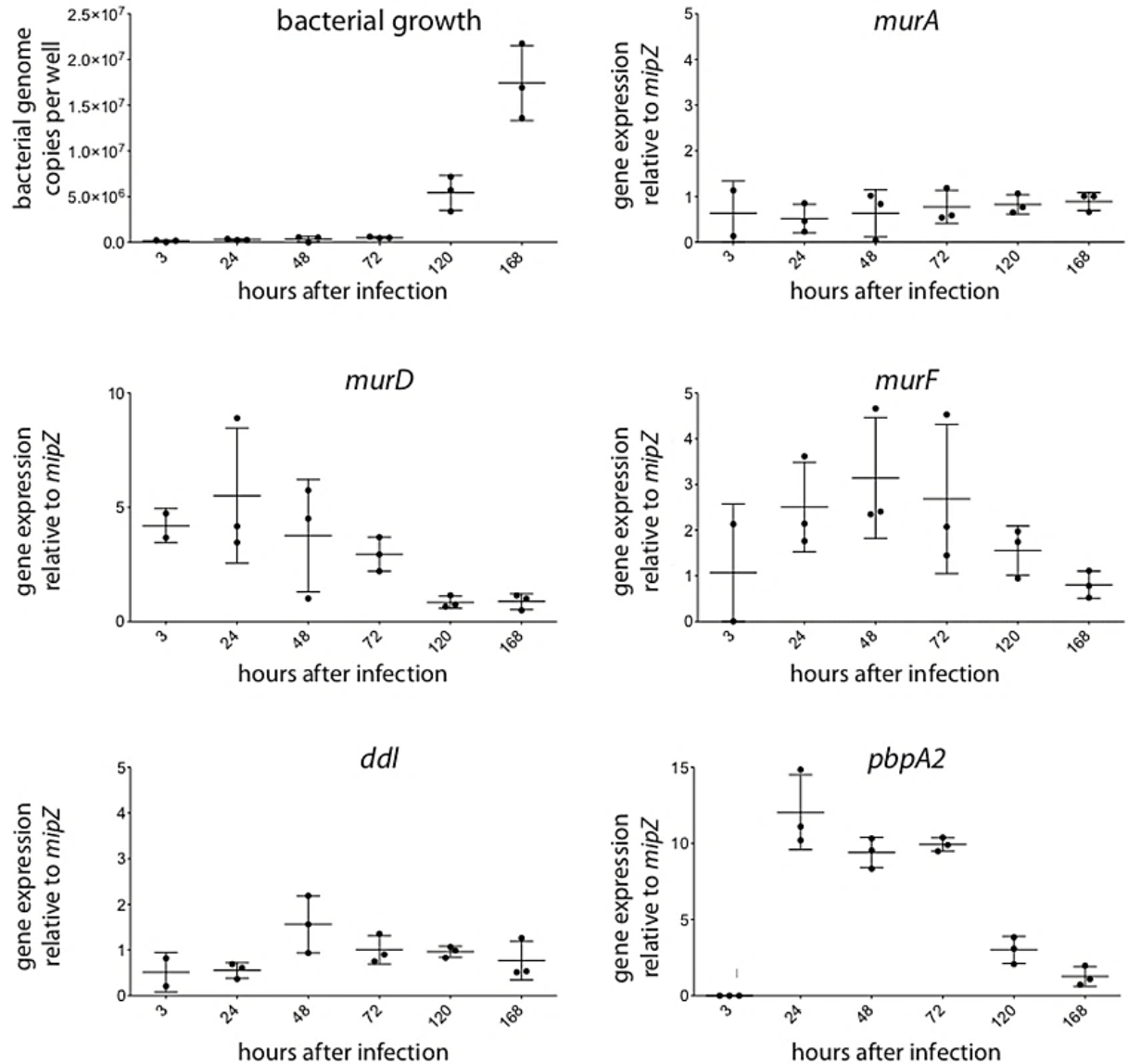


Figure 21. The expression of genes in the peptidoglycan synthesis pathway for *Orientia*

Bacterial genome copy number or relative gene expression of bacteria grown in mouse fibroblast L929 cells over 7 days. The relative expression level was determined by qPCR and normalised to the housekeeping gene *mipZ*. The graphs show each individual data point, as well as the mean and standard deviation.

This work was done by Suparat Giengkam.

4.3.c Drug sensitivity

Drug sensitivity can show if specific components of the peptidoglycan pathway are active. Also, if these components result in a lack of peptidoglycan synthesis, are bacteria able to survive and grow? To answer this, drugs which specifically target the cell wall were treated with *Orientia* to determine the effect of the drug on bacterial growth (**Figure 22, B-E**). The growth of *Orientia* was quantified after treatment with chloramphenicol, which inhibits protein synthesis at the translational level and is used to clinically treat scrub typhus along with doxycycline. This was compared with bacterial growth in the presence of d-cycloserine and phosphomycin. D-cycloserine targets the Alr/Ddl pathway, which produces the double D-alanine residues which are integrated into the peptide chain. Phosphomycin targets MurA which catalyses UDP N-acetylglucosamine to UDP N-acetylglucosamine enolpyruvate, in a condensation reaction. In all drug treatments, growth was impaired. The bacterial membrane structure became disfigured by d-cycloserine and bacteria become larger and rounder under the influence of phosphomycin. Recovery of *Orientia* growth treated with D-cycloserine was tried with the addition of exogenous D-Alanine but this was not successful (**Figure 22, C**).

Orientia is reported to be insensitive to penicillin, which was a key point used in the argument for it not synthesising peptidoglycan [231]. To corroborate this, the growth of *Orientia* was measured during treatment of β -lactam antibiotics. Alternative antibiotics were chosen from the carbapenem group, which target DD-transpeptidase PBPs. of those tested, imipenem and meropenem are highly resistant to β -lactamase and mecillinam exclusively targets PBP2. All carbapenem antibiotics presented no effect on *Orientia* growth. Even when bacteria were treated with penicillin plus the β -lactamase inhibitor, tazobactam, the antibiotic did not strengthen in effect. To investigate whether PBP2 and PBP3 are resistant to beta-lactam antibiotics in *Orientia*, active site residues were aligned from various bacterial species (**Figure 23**). Just one residue from PBP2 and

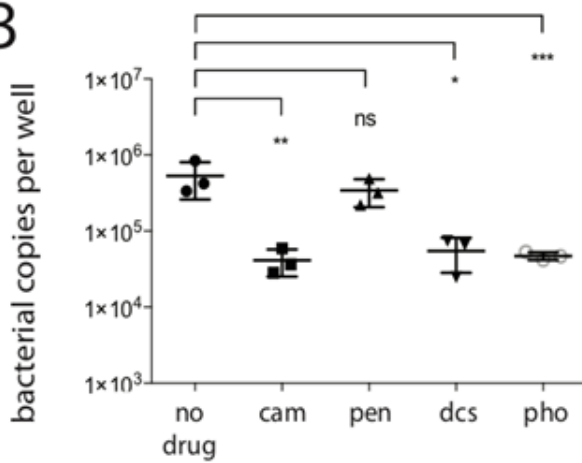
PBP3 were found to be mutated in *Orientia* which may explain why the bacteria are resistant to β -lactams even though peptidoglycan can be synthesised (alignment done by Jeanne Salje).

Taken together, growth of *Orientia* is sensitive to peptidoglycan targeting drugs, except for beta-lactams, indicating that the cell wall structure is necessary for division and cell shape. Furthermore, *Orientia* is insensitive to β -lactam antibiotics as previously reported but still synthesises peptidoglycan.

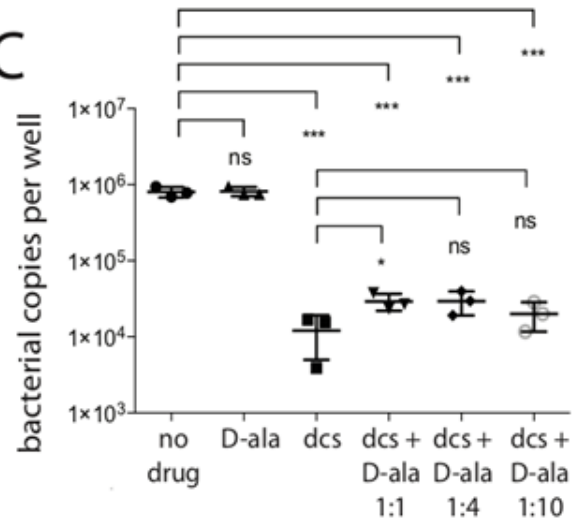
A

Drug or Enzyme	Molecular Target
chloramphenicol (cam)	50s subunit of ribosome
penicillin G (pen)	PBPs
D-cycloserine (dcs)	Alr and Ddl
phosphomycin (pho)	MurA
meropenem (mer)	PBPs
imipenem (imi)	PBPs
mecillinam (mec)	PBP3
tazobactam (taz)	β -lactamase
lysozyme (lys)	MurNAc-GlcNAc bond
mutanolysin (mut)	MurNAc-GlcNAc bond

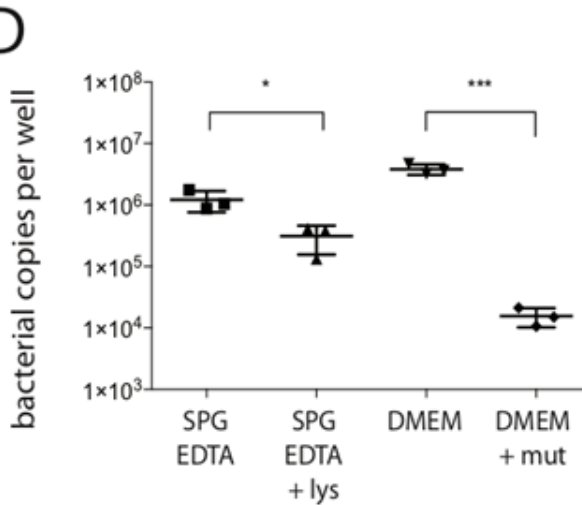
B



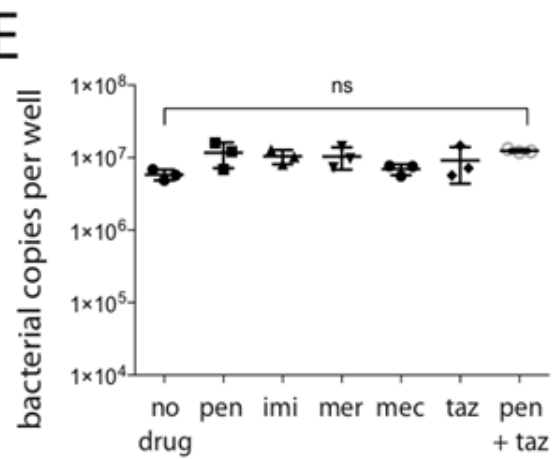
C



D



E



F

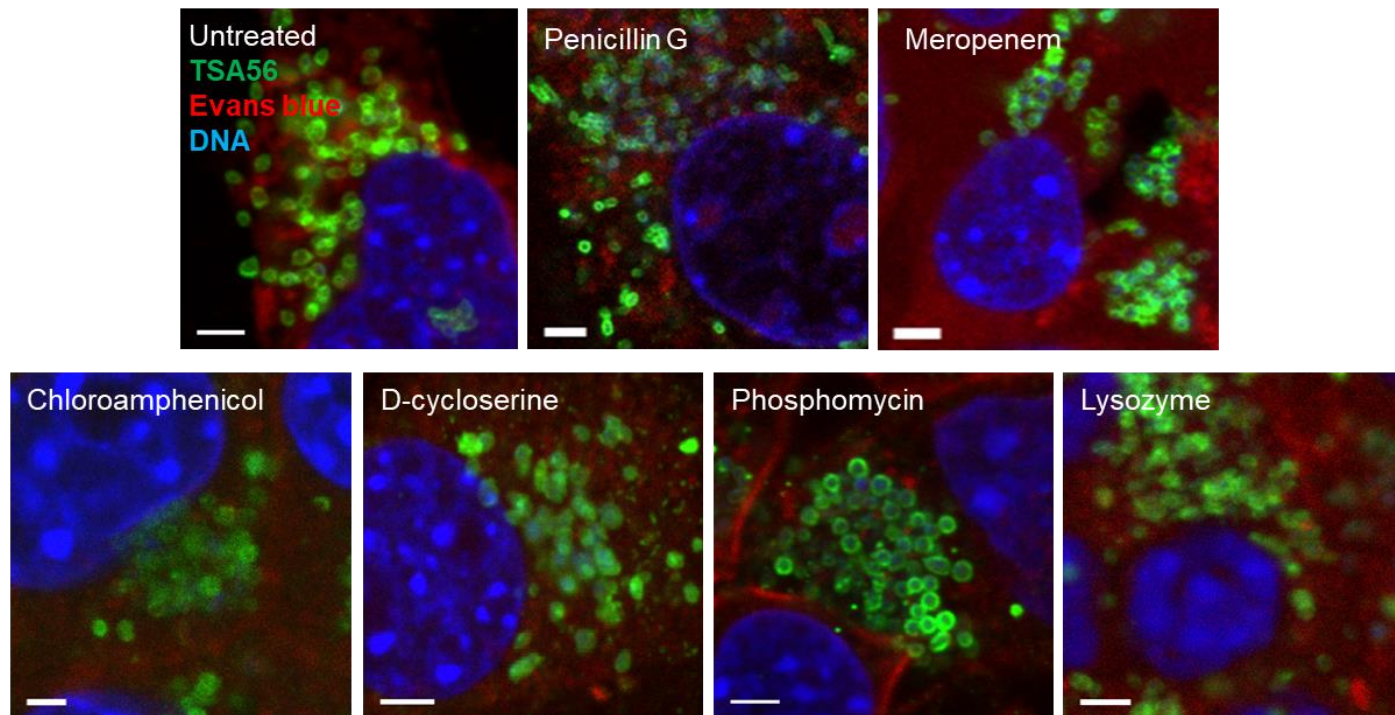


Figure 22. *Orientia* treated with cell wall targeting drugs

A: A table of the drugs or enzymes used in this study with their molecular target.

B-E: Bacteria were grown in L929 cells for 7 days and treated with various drugs. The copy number was then determined by RT-qPCR using triplicates for each condition. Graph shows each individual data point, as well as the mean and standard deviation. Statistical significance was determined using an unpaired t test analysis. P values are illustrated as follows: ns ($P > 0.05$); * ($P \leq 0.05$); ** ($P \leq 0.01$); *** ($P \leq 0.001$). Work done by Suparat Giengkam.

F: Drug sensitivity in *Orientia* to cell wall targeting drugs. Intracellular bacteria were treated with drugs for 24 hours and then fixed and labelled with TSA56 for immunofluorescence microscopy. Drug concentrations are as follows: chloramphenicol 100 ug/ml; penicillin G 150 ug/ml; D-cycloserine 250 ug/ml; phosphomycin 40 ug/ml; lysozyme 5 ug/ml; meropenem 10 ug/ml.

Scale bar = 5µm

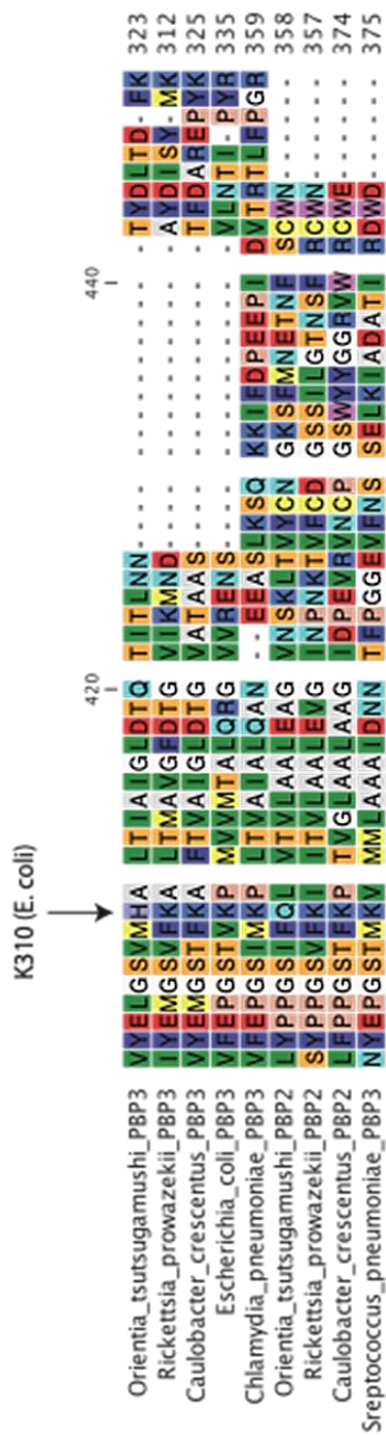


Figure 23. Protein alignment of PBP2 and PBP3 from bacterial species

The conserved K310 active site in diverse bacterial species. The K310 position for PBP3 is K but for *Orientia* it has changed to H. The K310 position for PBP2 is K but for *Orientia* it has changed to Q.

Work done by Jeanne Salje using cluster omega.

4.3.d Glycosyltransferase activity

Orientia cannot produce class A PBPs which are the bifunctional enzymes that are known to possess both transpeptidase and glycosyltransferase activity. *Orientia* only encodes class B PBPs which only possess transpeptidase activity. Without any class A PBPs is the glycan backbone of peptidoglycan in *Orientia* actually polymerised? To investigate this, enzymes lysozyme and mutanolysin were used to measure the sensitivity against *Orientia* growth. Lysozyme is an antimicrobial, found naturally in tears and mucus in animals. It catalyses the hydrolysis of the β 1,4 linkage between lipid II precursors. Mutanolysin, produced by Gram positive *Streptomyces globisporus*, also cleaves the β 1,4 linkage between lipid II precursors by its muralytic activity. Therefore, sensitivity to these would indicate the presence of a β 1,4 linkage between the lipid II precursors.

Both enzymes were able to cause the lysis of *Orientia* and prevented growth (**Figure 22, D, F**). This indicates that there is polymerisation between MurNAc and GlcNAc but how this polymerisation can occur is unknown.

4.3.e EDA

D-amino acid derivatives have been developed which can be incorporated into peptidoglycan, as described in chapter 2. These can be used to visualize peptidoglycan labelling through live or fixed imaging. These probes may be mono-peptide or dipeptide probes of D-alanine [223] [237] [224]. Ethynyl-D-alanine (EDA), an azide D-alanine dipeptide probe, was used to label peptidoglycan labelling in *Orientia* in a click chemistry reaction. EDA was able to label *Orientia* successfully and presented labelling all over the surface (**Figure 24**), indicating a peptidoglycan structure in the cell wall, unlike the septum labelling imaged in *Chlamydia* [223]. Further proving that EDA was taken up during peptidoglycan synthesis, cell wall targeting drug treatment with bacteria showed a lack of fluorescence after probe incorporation. Although the background of labelling is high, this was not seen in uninfected cells also labelled with the probes. The reason for this high background may be due to low peptidoglycan abundance or shed peptidoglycan in host cells.

It is not known exactly how EDA is incorporated into the peptidoglycan structure in *Orientia*. The integration of D-alanine occurs in the peptide chain by DD-transpeptidase activity, so EDA is expected to enter nascent peptidoglycan structure in this manner. However, EDA could also be incorporated during peptidoglycan remodeling in the cell wall again by transpeptidase activity.

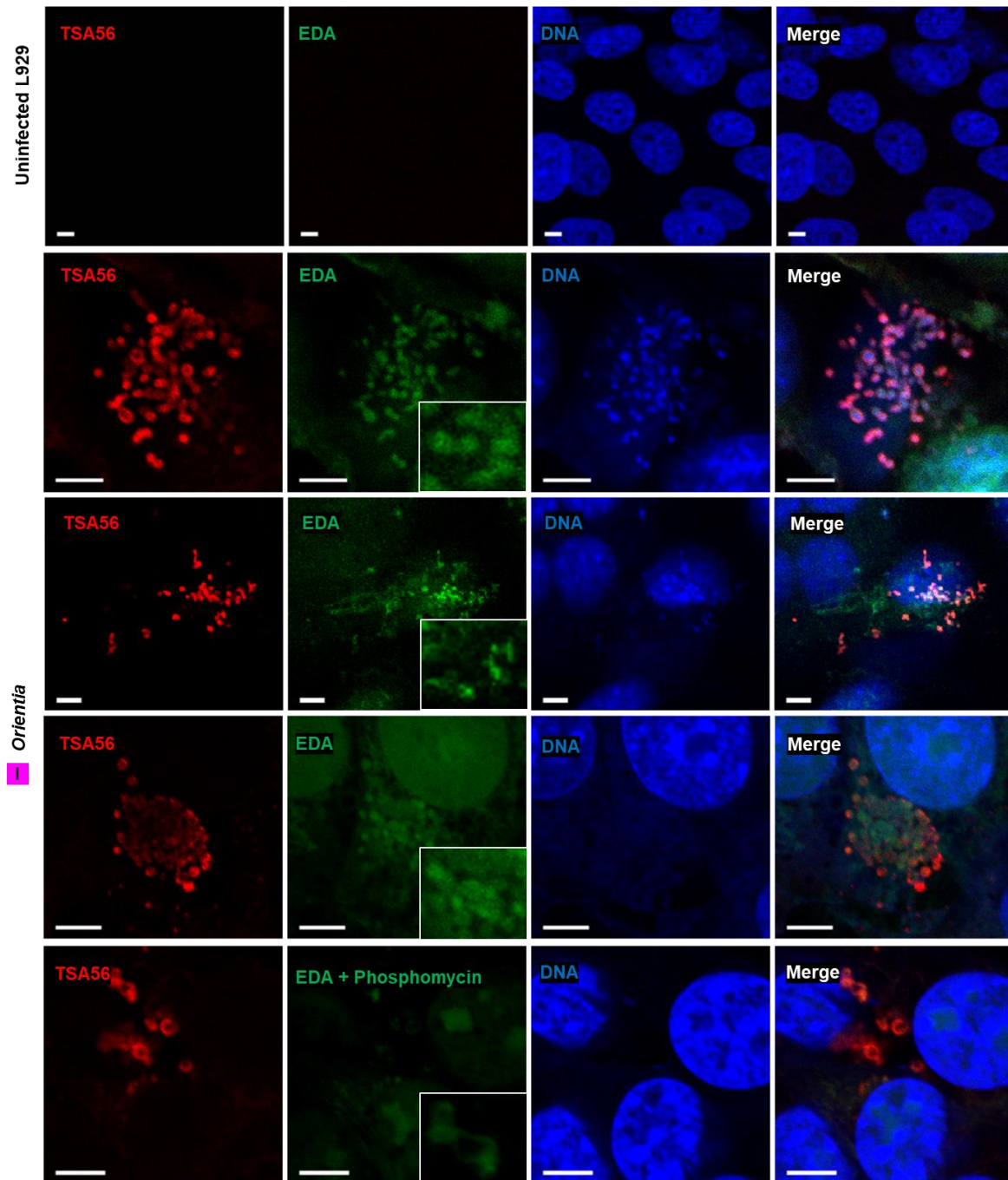


Figure 24. *Orientia* labelled with peptidoglycan probe EDA

EDA was added to L929 cells infected with *Orientia* at day 3 for 4 hours. After this time, cells were fixed, and a click reaction was carried out to allow detection by fluorescence. Bacteria were also labelled with TSA56.

The top row shows EDA labelling in uninfected L929 cells to compare infected cells.

Phosphomycin targets MurA. I = intermediate peptidoglycan

Scale bar = 5µm

4.3.f HADA and HALA

HADA (7-hydroxycoumarin-3-carboxylic acid-3-amino-D-alanine) is also a fluorescent probe, but is comprised of one alanine attached to a fluorophore [224] (**Figure 14, C**). HADA can also be imaged through live or fixed microscopy. The incorporation of HADA in *Orientia* presented labelling all over the surface, indicating like EDA, peptidoglycan synthesis is occurring in the cell wall of the bacteria (**Figure 25**). HADA is also thought to be integrated into the peptide chain, like EDA, as single D-alanine.

The L-isomer form of HADA, HCC-amino-L-alanine (HALA) was also tested. Surprisingly, this probe managed to label a sub-population of *Orientia* and even the control bacterial species *E. coli* (**Figure 25**). Since we cannot be exactly sure how HADA is taken up during peptidoglycan synthesis, HALA may also be incorporated in some way.

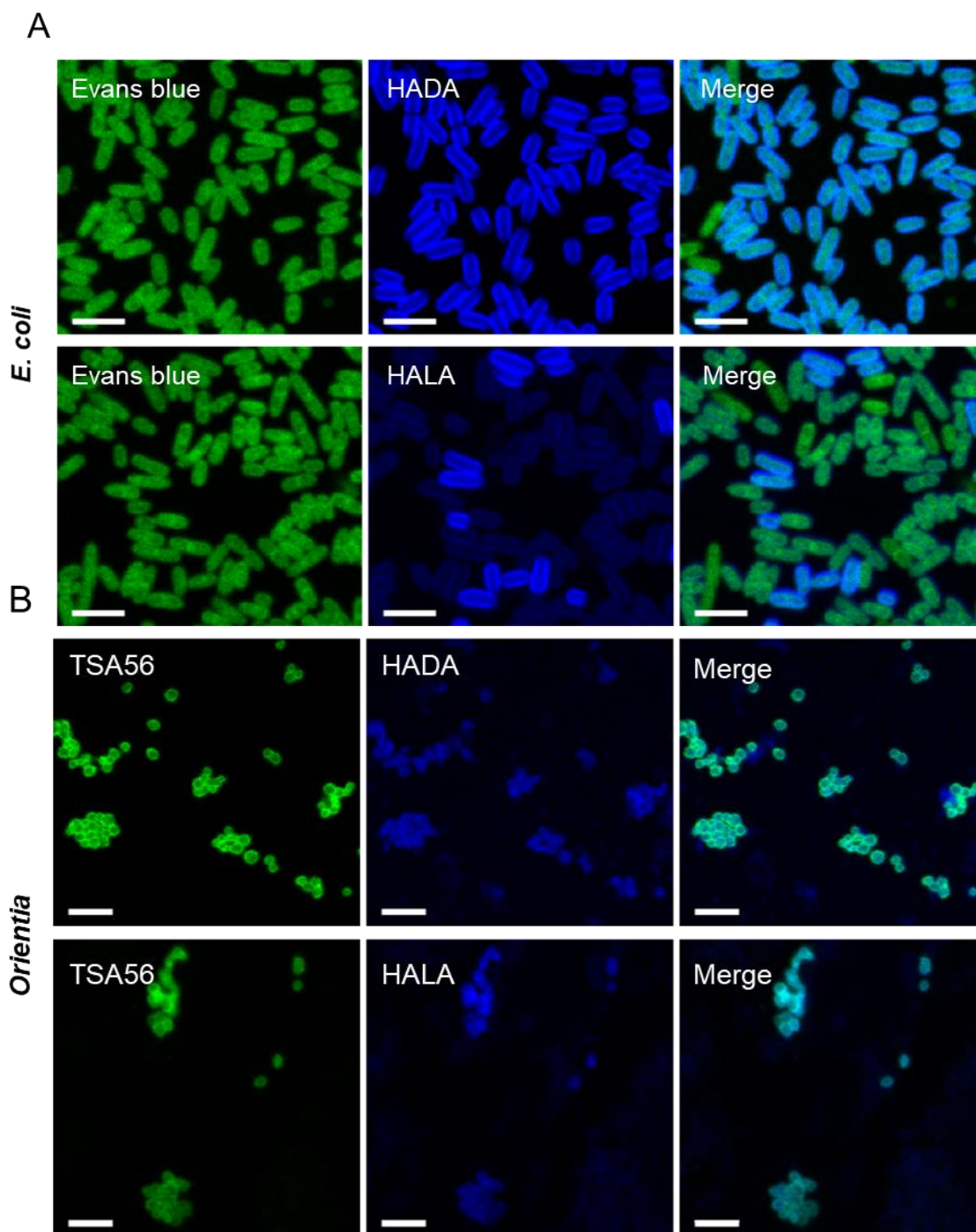


Figure 25. Fluorescence microscopy of fluorescent probes D-alanine HADA and L-alanine HALA.

A: HADA and HALA labelling of *E. coli* with evans blue membrane label.

B: HADA and HALA labelling of *Orientia* with TSA56 antibody.

Scale bar = 5µm

4.3.g Response to osmotic stress

Bacteria can withstand osmotic pressure due to the integrity of their cell wall, particularly by the presence of peptidoglycan in the periplasm. Obligate intracellular bacteria live in an osmotically protected environment of the host cell cytoplasm. Due to this, there is a reduced need for peptidoglycan in their cell wall, especially since peptidoglycan is recognized immunogenically by the host NOD1/NOD2 pathway. This may be why obligate bacteria such as *Orientia* and *Chlamydia* may have reduced the synthesis of peptidoglycan during their life cycle.

To verify if *Orientia* has a cell wall structure strong enough to confer osmotic protection, the bacteria were exposed to buffers to test its vulnerability. Purified *Orientia* did not lyse when in the presence of phosphate buffered saline (PBS), sucrose phosphate glutamate (SPG) buffer or water. When resuspended in water, bacteria did become larger but did not lyse, indicating that it must have a peptidoglycan-like structure which provides mechanical protection. Bacteria resuspended in PBS and water presented a slightly different phenotype from bacteria in SPG, as they become less smooth. If peptidoglycan was in fact not present, the bacteria would have lysed in water and PBS treatment due to the lack of protection in a high osmotic environment.

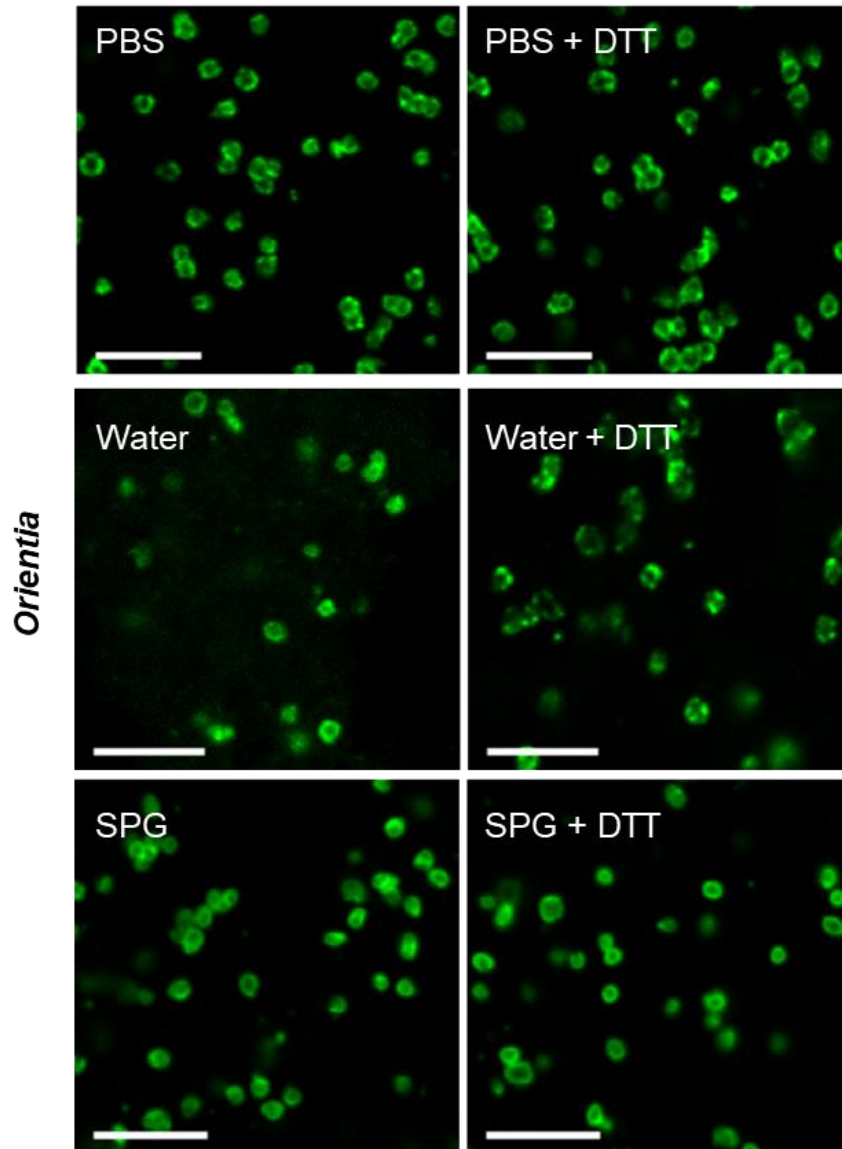


Figure 26. Testing the structural rigidity of *Orientia* by immunofluorescence microscopy

Bacteria were treated with various buffers such as PBS, water and sucrose phosphate glutamate solution, to test cell wall support. *Orientia* were then fixed and labelled with TSA56.

Scale bar = 5µm

4.3.h Cross-linked TSA56 in the outer membrane of *Orientia*

Results here show that there is clearly some form of peptidoglycan in the cell wall of *Orientia* but what else is maintaining the structure of the cell wall? Usually in Gram negative bacteria, the presence of LPS provides structural integrity. *Orientia* does not have LPS in its cell membrane, therefore there may be another component alongside peptidoglycan providing support. *Orientia* possesses a protein called TSA56 which is highly abundant on its outer membrane and this contains three conserved cysteine residues. The cell wall of *Chlamydia* is known to consist of major outer membrane proteins (MOMPs) which are stabilised by disulfide bonds and form a cross-linked network along the membrane [235] [238]. This led to the hypothesis that instead of LPS, TSA56 proteins may also crosslink along the membrane, held by disulfide bonds between cysteine residues.

To investigate this, DTT, a redox reducing agent was used. Purified bacteria were resuspended in SPG and PBS buffers containing DTT and imaged by microscopy with TSA56 antibody labelling (**Figure 26**). Although bacteria remained intact under DTT conditions, bacteria exposed to water and DTT presented disfigured cell membranes with variable TSA56 antibody labelling. This suggests that the double threat of water and DTT on the cell membrane, rendered *Orientia* more vulnerable as the cell membrane became weaker. *E. coli* was used as a control and did not experience the same changes as *Orientia* which suggests that the cross-linked surface protein in *Orientia* was specifically involved.

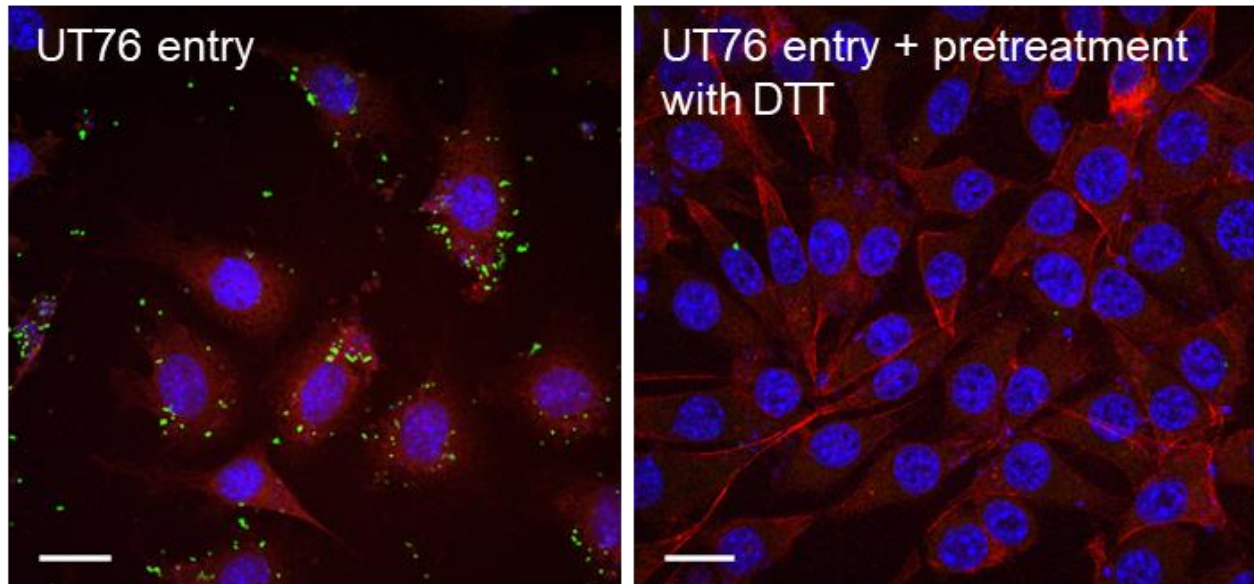
TSA56 is known to aggregate. Reducing agent, β -mercaptoethanol, was used with *Orientia* to show the lack of aggregation by western blot analysis (**Figure 27, C**). This was done by Suparat Giengkam.

It is known that TSA56 is a key surface protein and it interacts with host receptors to initiate bacterial entry. If this is true, how would entry be affected if the protein becomes destabilised by

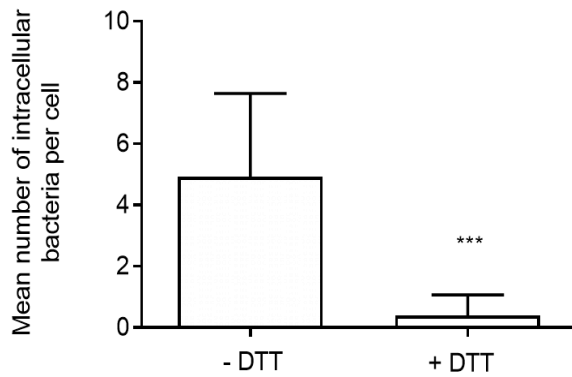
DTT? This was investigated by pre-exposing purified *Orientia* to DTT then washing bacteria in cell culture media before infecting host cells (**Figure 27**). Compared to bacteria which were not treated with DTT, the DTT treated *Orientia* presented a much lower entry number. This indicates that without a cross-linked TSA56 to interact with host fibronectin, entry is extremely reduced.

Taken together these results support the hypothesis that *Orientia* has a stable disulfide cross-linked membrane composed of TSA56 surface proteins which vital for infection and with the presence of a peptidoglycan-like structure, essential for structural support.

A



B



C

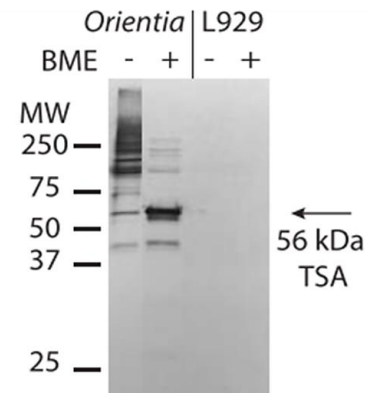


Figure 27. Immunofluorescence microscopy of *Orientia* treated with DTT in L929 cells

A: Bacterial entry without any treatment was compared to bacterial entry after 1mM DTT treatment, which was then washed off prior to infection.

B: *Orientia* were labelled with TSA56 and entry was quantified manually 24 hours after mock treatment or pre-treatment with DTT. 100 host cells were randomly selected, imaged, and manually counted. The mean and standard deviation is shown, with statistical significance determined using an unpaired t test analysis. P values are illustrated as follows: ns ($P>0.05$); * ($P0.05$); ** ($P0.01$); *** ($P0.001$).

4.4 Discussion

Orientia has a peptidoglycan-like structure but this not the classic peptidoglycan known in model systems such as *E. coli*. The peptidoglycan in the cell wall is much more reduced in abundance and its synthesis may be unconventional.

This is shown firstly by the identification of *mesoDAP*. This could be detected but the abundance was very low. Some of the genes for its synthesis are absent in *Orientia* and it is likely that alternative pathways are involved.

Measurement of gene expression of the genes involved in peptidoglycan synthesis over 7 days of infection was achieved by qRT-PCR. Expression varied according to the gene but during early stages of infection, *murD* and *murF* presented high levels of expression. This may indicate that the buildup of peptidoglycan precursors is needed as the bacteria prepare for division. This may be further explored in the future by microscopy by the production of RNA FISH probes against *mur* genes.

Further evidence of the presence of peptidoglycan was shown by treatment of drugs which target the cell wall. All carbapenem antibiotics presented no effect on *Orientia* growth which may be due to the mutated position at K310 in PBP3 and PBP2. However other drugs such as phosphomycin, d-cycloserine and lysozyme all affected the cell wall of *Orientia* as well as the viability to the same level as chloramphenicol treatment.

Orientia is missing the amino acid racemases Alr. The presence of the D-form amino acids in the peptide chain of peptidoglycan is unique and usually racemase activity is used to convert the L-form to the D-form amino acid. The D-isomer amino acids are not present in proteins, and the D-alanine dipeptide is unique to peptidoglycan. Treatment of *Orientia* and D-cycloserine with high levels of D-Alanine could not help bacteria recover and synthesise peptidoglycan which was shown to occur in *Chlamydia* [239]. *Orientia* does have Ddl suggesting that a D-ala-D-ala

dipeptide is present and incorporated into a growing pentapeptide side chain. An alternative enzyme is likely to perform the racemase activity, or the synthesis of the peptide chain may rely on Ddl.

Orientia does not encode class A PBPs which exhibit glycosyltransferase activity but still appears to have a polymerised glycan backbone as lysozyme and mutanolysin were able to affect bacterial viability and structure. Loss of class A PBP in various bacteria species such as *E. coli* have proven lethal [191]. However, it has been shown in *Bacillus subtilis*, which encodes 4 class A PBPs, and with the removal of all 4, peptidoglycan is still synthesised with minor structural differences and slow growth [240]. Furthermore, *Enterococcus faecium* also displayed glycan chain polymerisation after deletion of all 3 of its class A PBP genes [241]. Taken together, this indicates that lacking class A PBPs in bacteria does not mean necessarily mean no peptidoglycan structure in the cell wall.

But how may the peptidoglycan glycan backbone be joined together? One candidate for glycosyltransferase activity in *Orientia* may come from the SEDS family. Some SEDS proteins have dual roles as many are involved in peptidoglycan growth and maybe also peptidoglycan synthesis. For example, FtsW is thought to transport lipid II across the membrane. MurJ also supposedly acts as a lipid II flippase [181]. Evidence of dual activity has opened the door to the possibility of other components performing multiple activities including glycosyltransferase activity [195]. SEDs protein RodA is known to be essential for bacterial viability and has been shown to exhibit glycosyltransferase activity in *B. subtilis* [179]. Depletion of RodA in various bacterial mutant species lacking class A PBPs, arrested growth completely [242]. This suggests that RodA may provide glycosyltransferase activity for peptidoglycan synthesis during elongation. FtsW may also provide glycosyltransferase activity during cell division. If this is true, antibiotics could possibly be generated to target SEDS in bacterial species where class A PBPs are nonexistent.

The integration of fluorescent D-alanine probes was used to show cell wall labelling by microscopy. Clickable EDA and EDA-DA probes were able to label intracellular *Orientia* and after phosphomycin and d-cycloserine treatment, both probes labelled *Orientia* less efficiently. However, EDA and EDA-DA labelling was not as intense as labelling in positive control *E. coli*. This may indicate that the abundance of peptidoglycan in *Orientia* is low or probe addition was not incorporated at the optimal stage of peptidoglycan synthesis during infection which may explain why the background labelling of EDA and EDA-DA was very high in infected cells only. Furthermore, *E. coli* presented sepal peptidoglycan labelling (also seen in *Chlamydia* [225]) and in *Orientia*, the entire body of the bacteria became labelled. Occasionally, localised cell wall labelling was visualised, but this was hard to find. The incorporation of these probes is not known exactly so labelling may differ in bacterial species.

HADA and HALA both managed to label the cell wall of *Orientia*, although the population labelled with the latter probe was much smaller. The probes may be taken up via the DA-DA pathway by Ddl during peptide chain formation, but it cannot be ruled out that other sites of incorporation may occur such as during remodeling of peptidoglycan by class B PBP activity, LD-transpeptidase activity (although *Orientia* lacks LD-transpeptidases) or uptake by Ddl and MurF. Enzymes which are responsible for D-alanine addition into peptidoglycan may not discriminate the L isomer from the D isomer.

D-alanine probe incorporation may differ for all species of bacteria and there may be multiple processes which may incorporate D-alanine. Labelling of HADA, HALA, EDA and EDA-DA was not consistent in every experiment which may mean host or bacterial factors effect labelling. This can include host cell confluence, host cell line, bacterial infection number, time probe is added after infection and media used for probe labelling.

Peptidoglycan provides protection against osmotic stresses. *Orientia* is free in the osmotically protected cellular environment during its intracellular life cycle in the host cell. This would be one

reason for the bacteria not needing peptidoglycan in its cell wall. Since *Orientia* did not lyse in PBS or water, this indicates that there is a protective structure in their cell wall. The morphology of bacteria in PBS and water presented a rougher cell envelope compared to bacteria treated with SPG. *Orientia* were also found to grow better after storage in SPG rather than PBS or water, which shows that PBS and water exert high osmotic pressure and this may damage the cell envelope [138].

Orientia does not have LPS on its outer membrane. Instead, surface protein TSA56 is abundant which is vital for entry in host cells and it contains 3 cysteine residues which have a disulfide linked composition on the outer membrane of the bacteria. As shown by treatment of bacteria with reducing agent DTT in PBS, SPG and water, bacteria became more fragile. This shows that in addition to a peptidoglycan-like structure in the cell wall, this cross-linked outer membrane provides support and structural aid to the bacteria similar to the major outer membrane proteins of *Chlamydia* [243]. The cross-linked outer membrane of TSA56 is also vital for infection as shown by treatment of *Orientia* with DTT before entry. Entry may not occur without the crosslinked TSA56 as the proteins may need to be aggregated or composed in a more sophisticated structure with other surface proteins for fibronectin binding to initiate entry.

In this chapter, I show that there is a peptidoglycan-like structure in the cell wall of *Orientia*. This structure is essential for bacteria growth, structure and osmotic protection. Furthermore, *Orientia* has a network of disulphide cross-linked outer membrane proteins composed of TSA56 and possibly other proteins.

Like *Chlamydia*, the theory that this organism lacks peptidoglycan has again been disproved for *Orientia*. What does this mean for other Rickettsiales species? *Rickettsia* species are understood to contain a classical peptidoglycan structure but for other less known species, this is unclear. It will be interesting to apply the same methodology here to Rickettsiales species with uncharacterised peptidoglycan status.

Chapter 5

Peptidoglycan in Rickettsiaceae and Anaplasmataceae

This research is currently being prepared for publication.

5.1 Introduction

Both *Orientia* and *Chlamydia* are obligate intracellular bacteria that have now been shown to have low levels of a peptidoglycan-like structure. Interestingly, a comparison of the genes that are present or absent in these organisms showed that both retained many genes involved in peptidoglycan biosynthesis, but both lacked any genes for bifunctional class A PBPs. This led to an extensive bioinformatics analysis to study the presence and absence of peptidoglycan related genes in all major known obligate intracellular bacteria to see if this was indicative of a larger pattern. **Figure 28. A summary of the key peptidoglycan gene homologs present or absent in bacterial species** [1] shows a summary of the main genes involved in peptidoglycan biosynthesis and their presence in eleven obligate intracellular bacteria and a further 7 related free-living bacteria [1]. This work was performed by collaborators and other members of the lab.

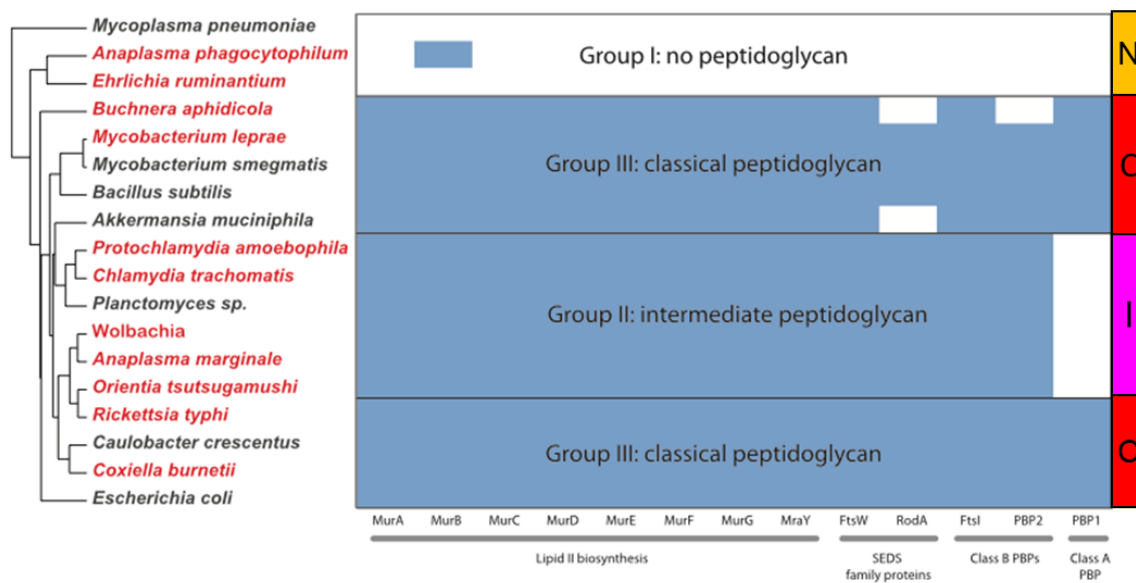


Figure 28. A summary of the key peptidoglycan gene homologs present or absent in bacterial species [1]

Species in red are obligate intracellular bacteria and free-living bacteria are in black. The status of peptidoglycan in these species are predicted on the right. Colour codes on the right will be used in this thesis to distinguish each group based on the bioinformatic gene predictions (orange: no peptidoglycan present, red: classical peptidoglycan present and pink: intermediate peptidoglycan).

Based on this analysis, bacteria can be divided into three groups. One group lacks almost all genes required for peptidoglycan biosynthesis and would be predicted to lack any peptidoglycan. One group contains all the key genes involved in peptidoglycan biosynthesis. Finally, one group contained almost all genes involved in peptidoglycan biosynthesis but lacks any class A PBPs. This last group includes *Orientia tsutsugamushi* and *Chlamydia* spp. and other Rickettsiales organisms including *Wolbachia* and *Anaplasma marginale*. This led to the prediction that these organisms might all make a similar peptidoglycan-like structure and this motivated the work in the current chapter.

The bacterial species in Rickettsiaceae and Anaplasmataceae families all infect various vectors and exhibit different cell cycles in different host cells. This will result in differences in the selective pressure for or against peptidoglycan.

Whilst the genes for peptidoglycan synthesis are known in the genome of these species, it is not clear if they are expressed and if peptidoglycan is being synthesised. The difficulty in exploring this arises from the fact that these species can only be grown inside host cells and many species remain genetically intractable. This chapter will concentrate on representatives from each of these predicted peptidoglycan groups and their ability to synthesise peptidoglycan. The presence of peptidoglycan in many Rickettsiales species has never been shown so a few species were investigated from each family.

5.1.a *Rickettsia*

Rickettsial bacteria have a complete set of genes for peptidoglycan synthesis and the classic peptidoglycan structure has already been shown. The presence of peptidoglycan has been shown by electron microscopy for SFG and TG species, showing the similarities of structure of the cell wall with well-known *E. coli* [230]. The addition of penicillin, a peptidoglycan targeting drug, with rickettsia species, creates spheroblasts and inhibits growth. This is further proof that peptidoglycan is present [231]. *Rickettsia prowazekii* has also been shown to be sensitive to lysozyme and other peptidoglycan targeting drugs [155].

5.1.b *Anaplasma marginale*

The genome of *A. marginale* shows most genes are present for peptidoglycan synthesis. Most genes are present for meso-diaminopimelic acid (*meso*DAP) but *lysA* which is required for lysine biosynthesis is missing [90]. However, like *Orientia*, *A. marginale* is missing class A PBPs [1]. This indicates that the bacteria, do not possess the activity of bi-functional PBPs. Only transpeptidase activity is present through class B PBPs so how the glycan chains are bound is unknown.

5.1.c *Anaplasma phagocytophilum*

Unlike *A. marginale*, *A. phagocytophilum* do not possess most of the genes for peptidoglycan synthesis [139] [1] as only *murB* is present. From this genetic data, it can be assumed that *A. phagocytophilum* does not synthesise peptidoglycan. The cell wall is much weaker and with no peptidoglycan for structural support, the bacteria may have to compensate for this loss. *A. phagocytophilum* may instead be dependent on the integration of cholesterol into their membrane

although the bacteria lack the genes for cholesterol biosynthesis or modification [244]. The loss of the genes needed for lipid A synthesis may have occurred over time.

Mycoplasma are also known to lack peptidoglycan and this organism incorporates cholesterol into its membrane from the environment [245]. This may be for structural purposes, but cholesterol is also involved in many roles such as membrane and cellular functions. This may also be the case for *A. phagocytophilum* [139] [146]. A high cholesterol content may facilitate *A. phagocytophilum* infection in patients with HGA, especially since patients are usually older than 50 years of age. This indicates that high cholesterol diets in humans can cause them to be more susceptible to this bacteria species [244].

A. phagocytophilum infects monocytes or macrophages, unlike *A. marginale* which infects red blood cells. This difference may have resulted in selective pressure on only *A. phagocytophilum* to lose peptidoglycan.

5.1.d *Ehrlichia*

Ehrlichia cannot synthesise peptidoglycan based on genomic analysis, and like *A. phagocytophilum*, only peptidoglycan synthesising gene *murB* is present [1] [115]. *Ehrlichia* are known to be fragile and may compensate for the lack of physical integrity in their cell wall by also integrating cholesterol into the membrane [139]. As *Ehrlichia* infects monocytes, the genes needed for peptidoglycan synthesis may have been selected out over time to avoid recognition from toll-like receptors and nucleotide binding oligomerisation domain-containing intracellular protein receptors. This uptake of cholesterol into the bacterial membrane may be essential for host infection and other cellular functions necessary for bacterial survival [139]. Treatment with cholesterol extracting reagent, methyl β -cyclodextrin, results in *Ehrlichia* bacteria unable to attach or invade host cells [139]. As well as the lack of peptidoglycan, *Ehrlichia* do not synthesise LPS.

5.1.e *Wolbachia*

Similar to *Orientia* and *A. marginale*, *Wolbachia* encodes most of the genes essential for peptidoglycan synthesis but also lacks class A PBP transglycosylase activity.

What is already known about the cell wall of *Wolbachia*? Most genes needed for lipid II production are present in *Wolbachia* including the genes needed for *meso*DAP formation. Phosphomycin, inhibitor of MurA, can affect the growth of peptidoglycan in *Wolbachia* and the construction of the lipid II structure is vital for their growth and cell division [193] [141]. With no glycosyltransferase activity, a peptidoglycan structure composed by only transpeptidation of lipid II was hypothesised [246].

With no racemase for L-alanine to D-alanine conversion, *Wolbachia* may instead produce D-alanine via another metabolic pathway such as methionine biosynthesis which exhibit enzymes with dual activity, as shown in *E.coli* [141]. Also, lipoprotein AmiD in wMel strain *Wolbachia*, was found to cleave the MurNac-L-Alanine bond in lipid II so may help to recycle lipid II to avoid the immune response from the host [247].

5.2 Peptidoglycan in Rickettsiales: Objectives

Objective 1: To grow Rickettsiales species

- Approach: To develop protocols to grow *R. canadensis*, *A. marginale*, *A. phagocytophilum*, *E. chaffeensis* and *Wolbachia* in eukaryotic or insect cell lines and determine bacterial copy number by qPCR
- Expected outcome: Validated protocols for propagation and quantification of 5 rickettsial species

Objective 2: To label Rickettsiales species

- Approach: To optimise methods to label *R. canadensis*, *A. marginale*, *A. phagocytophilum*, *E. chaffeensis* and *Wolbachia* with fluorescent probes (clickable methionine and RNA FISH) for fixed microscopy imaging
- Expected outcome: Validated protocols and images of 5 rickettsial species labelled with clickable methionine and RNA FISH.

Objective 3: To measure NOD1 activation in response to Rickettsiales species

- Approach: To measure the host NOD1 response to *R. canadensis*, *A. marginale*, *A. phagocytophilum*, *E. chaffeensis*, *Orientia* and *Wolbachia* by adding heat inactivated bacteria to a NOD1 reporter cell line.

- Expected outcome: Bacterial species which do not synthesise peptidoglycan will not activate the host cell NOD1 response in contrast to those species which do have peptidoglycan.

Objective 4: To determine the sensitivity of Rickettsiales species to cell wall targeting drugs

- Approach: To analyse the effect of cell wall targeting drugs (phosphomycin, penicillin and d-cycloserine) on the viability of *R. canadensis*, *A. marginale*, *A. phagocytophilum*, *E. chaffeensis* and *Wolbachia* using qPCR-based growth measurements.
- Expected outcome: The growth of species which have peptidoglycan will be reduced. Species which do not synthesise peptidoglycan be unaffected.

Objective 5: To establish the subcellular localisation of peptidoglycan in Rickettsiales species

- Approach: To use D-alanine based peptidoglycan-specific chemical probes to label *R. canadensis*, *A. marginale*, *A. phagocytophilum*, *E. chaffeensis* and *Wolbachia* for fixed imaging, with and without cell wall targeting drug treatment. Peptidoglycan localisation by the probe will be compared to the localisation in control species (*E. coli* and *B. subtilis*)
- Expected outcome: Rickettsiales species which do not have peptidoglycan will not be labelled by the probe. If species do label, this is evidence of peptidoglycan in the cell wall, especially if blocked after the addition of a cell wall targeting drug.

Objective 6: To determine the sensitivity of Rickettsiales species to osmotic stress

- Approach: To isolate bacteria and measure the effect of different osmotic conditions on their morphology.
- Expected outcome: Rickettsiales species lacking the structural support of peptidoglycan are expected to be more sensitive to hypotonic buffers than those that synthesise a peptidoglycan cell wall.

Objective 7: To determine whether peptidoglycan in different Rickettsiales species contains a polymerized glycan backbone.

- Approach: To determine the sensitivity of Rickettsiales species to lysozyme and mutanolysin drugs as assessed by fluorescence microscopy.
- Expected outcome: Rickettsiales species that lack polymerized peptidoglycan will be insensitive to lysozyme and mutanolysin.

5.3 Results

5.3.a Propagation

All Rickettsiales species, except for *Orientia*, had not been grown before in this laboratory. Therefore, growth of all species had to be verified and quantified in various cell lines. *Anaplasmas*, *Ehrlichia* and *Wolbachia* were kindly given by Ulrike Munderloh (University of Minnesota) whilst *Rickettsia canadensis* was donated by Nancy Connell (Rutgers, University of New Jersey).

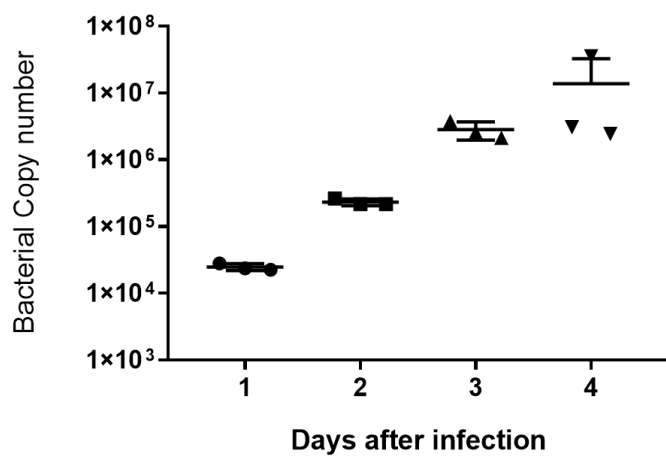
Bacteria were added to various cell lines and then quantified by qPCR against the 16s gene. *A. marginale* and *R. canadensis* were successfully grown in monkey endothelial Vero cells. *Ehrlichia chaffeensis* was grown successfully in Vero and dog macrophage DH82 cells (**Figure 29, C**). Growth for bacteria increased after inoculation and was able to increase up to day 4. However, for *Wolbachia* and *A. phagocytophilum*, growth was extremely slow. *A. phagocytophilum* were initially grown in human monocytic THP1 cells but growth was not consistent and failed to progress. Due to this, *A. phagocytophilum* were instead grown in human promyeoblast HL60 cells and were able to maintain steady but slow growth over time (**Figure 29, E**). Growth was also tested in endothelial RF6A cells which also proved successful (data not shown). *Wolbachia* are commonly grown in insect cell lines at 28°C. Growth was tested in *Aedes albopictus* C636 cells and fibroblast L929 cells. In both cell lines, growth was slow but consistent (**Figure 29 D**).

Although bacteria were grown successfully and used in experiments, further research needs to be carried out on bacterial propagation and purification. All bacterial species have been shown to grow in insect cell lines such as tick ISE6 cells [144, 151, 248-250], but this couldn't be achieved at this time. Also, lysis of host cells was achieved by the same technique used to lyse *Orientia*. This laboratory has tested extensively the best way to propagate and purify *Orientia* [138] for optimal propagation but these protocols may not be suitable for other Rickettsiales species..

Many species which are predicted to not have any peptidoglycan in their cell wall may be more fragile and sensitive so lysis techniques may damage the bacteria and therefore effect entry and growth. Although there are research papers which describe how species should be purified, there is no literature on tested techniques and results. Without this evidence, the way the bacteria are grown may not be optimal and may render experiments inconsistent. This issue will be addressed in future work.

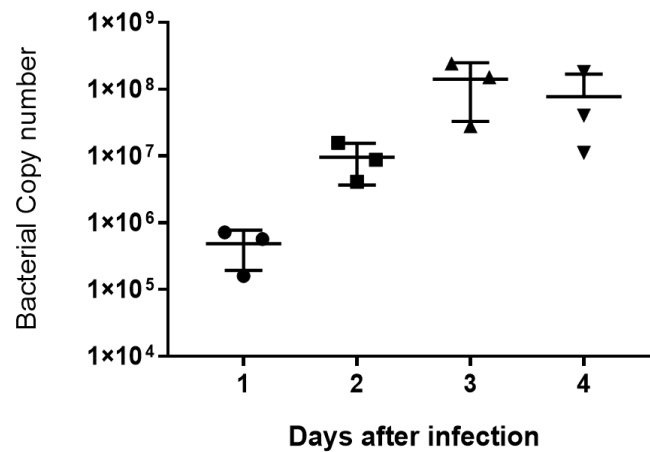
A

c *R. Canadensis*
VERO



B

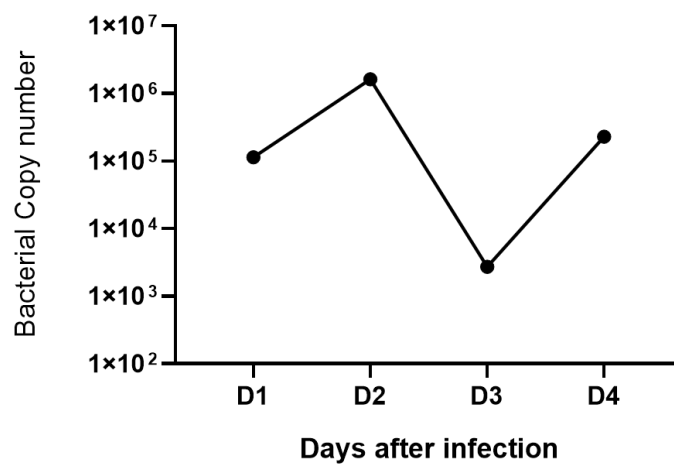
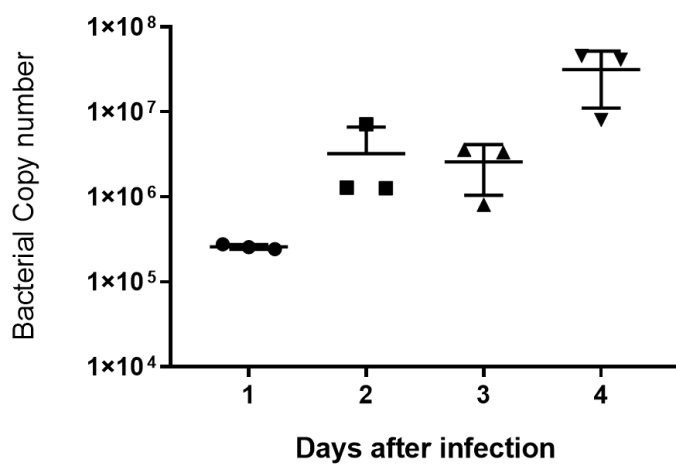
l *A. Marginale*
VERO



C

N *E. chaffeensis*

VERO



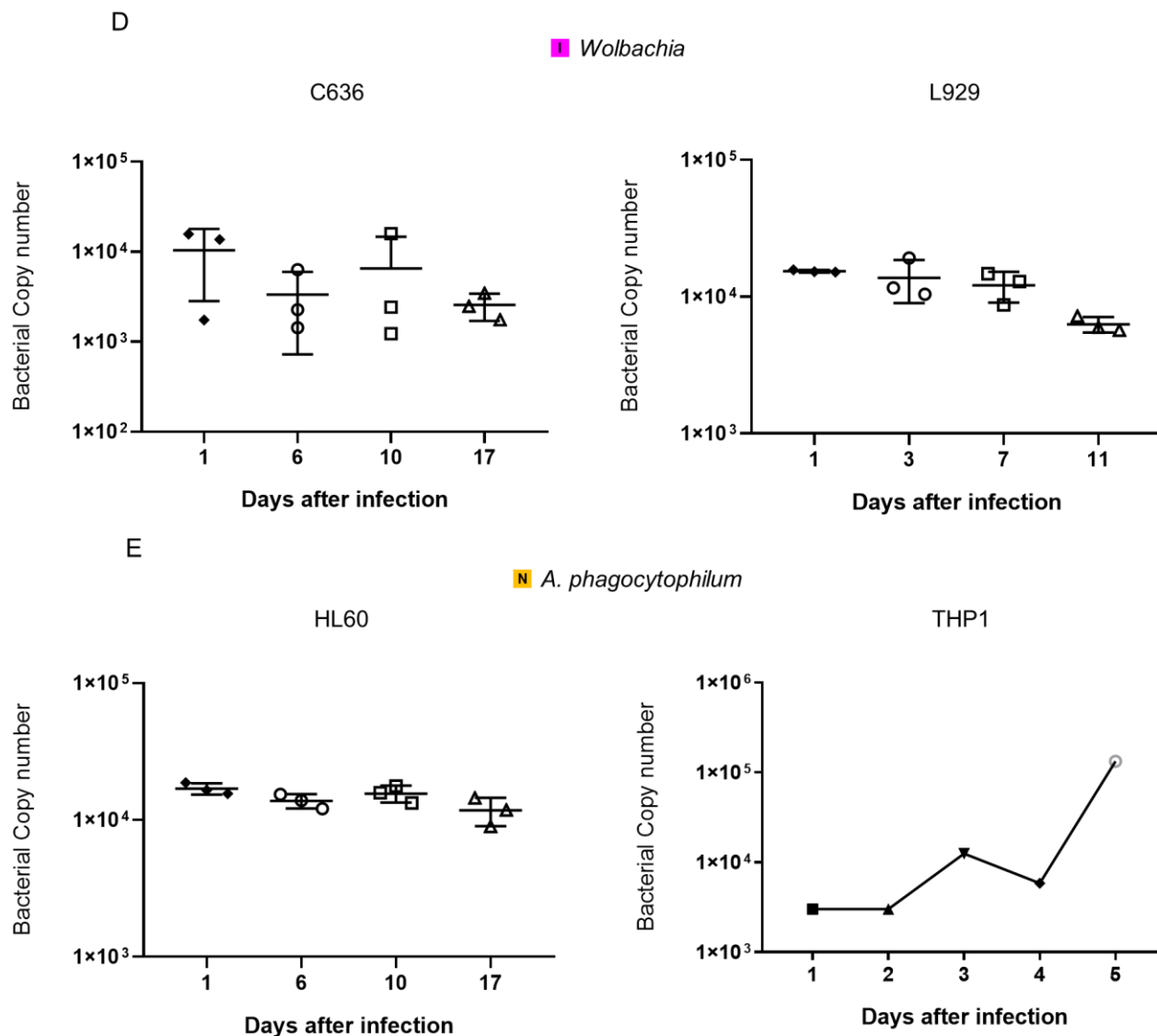


Figure 29. Rickettsiales growth in various cell lines

A: *R. canadensis* growth in vero cells over 4 days.

B: *A. marginale* growth in vero cells over 4 days.

C: *E. chaffeensis* growth in vero cells (left) and DH82 cells (right). The DH82 infection was not carried out in triplicate.

D: *Wolbachia* growth in C636 cells (left) and L929 cells (right).

E: *A. phagocytophilum* growth in HL60 cells (left) and THP1 cells (right). The THP1 infection was not carried out in triplicate.

All bacteria were grown in triplicate and measured from 16s copy number by RT-qPCR. The colour codes next to bacterial species indicates the peptidoglycan group they belong to based on gene presence.

C = Classical peptidoglycan, I = Intermediate peptidoglycan, N = No peptidoglycan

5.3.b Methionine

With no antibodies made for the multiple Rickettsiales species, unlike *Orientia*, labelling by immunofluorescence techniques was difficult. L-Homopropargylglycine (HPG) (ThermoFisher, USA) or L-Azidohomoalanine (Click chemistry tools, USA) probes used to label *Orientia* were also used to label Rickettsiales species successfully (**Figure 30**). The probes were added to infected cells 30 minutes prior to fixation. As the Anaplasmataceae species exist in two states during the infection cycle, there may have been an issue labelling the bacteria when not in active states. Bacteria were all labelled at day 3 of infection and this proved to show metabolically active bacteria. *A. phagocytophilum* and *Wolbachia* were also labelled successfully indicating that although growth is slow, bacteria are still metabolically active. The success of this allowed a reliable counter label to future labelling experiments.

5.3.c RNA FISH

With no antibody labelling and the fact that methionine labelling can be an issue if the bacteria are not active, another more reliable label was sought. Following the success of *Orientia*, the RNA fluorescence in-situ hybridisation (RNA FISH) probe was used to label Rickettsiales species. RNA FISH targeting 16s was used at 1ng/ul and successfully labelled all species at day 3 of infection (**Figure 31**).

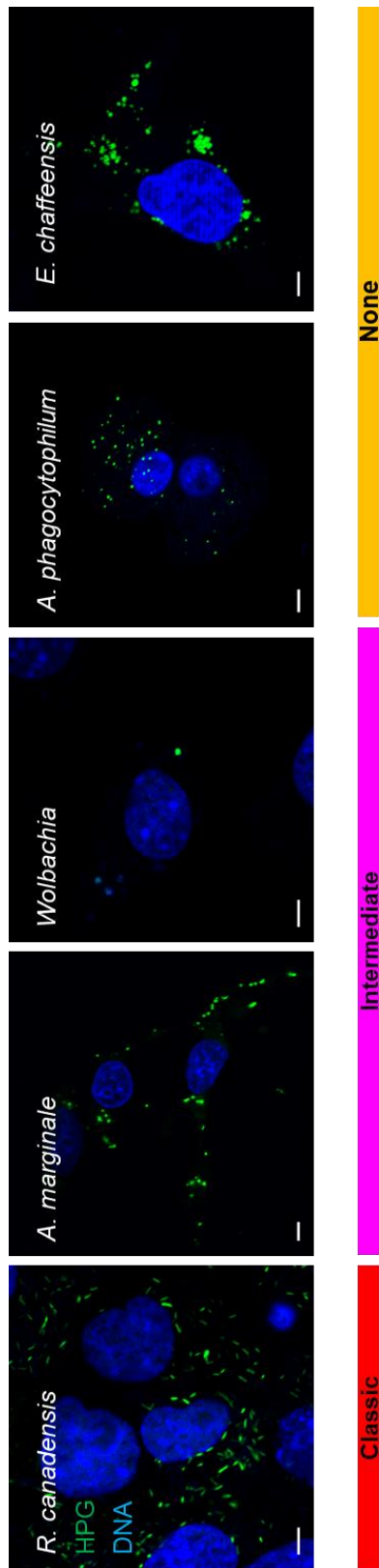


Figure 30. Methionine labelled intracellular Rickettsiales species

Methionine analog probe Click-IT™ L-Homopargylglycine (HPG) was used to label each bacterial species at various infectious stages, 30 minutes prior to fixation. Following fixation, a click reaction was carried out to allow detection by fluorescence microscopy. Scale bar = 5µm

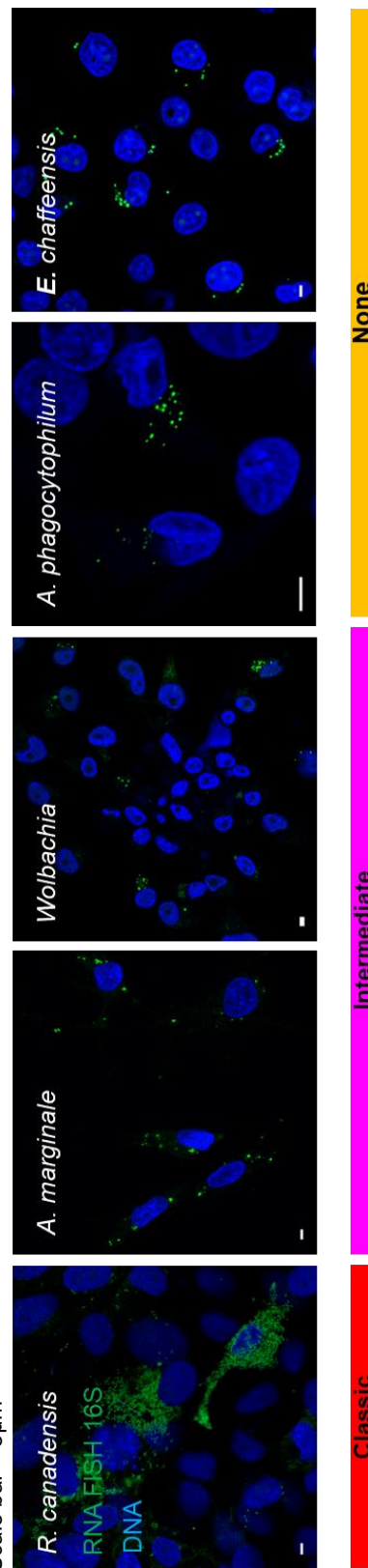


Figure 31. RNA FISH labelled Rickettsiales species

Bacterial infections were fixed and labelled with a fluorescent RNA fluorescence in situ hybridisation (FISH) probe specific for 16S. Scale bar = 5µm

5.3.d NOD1 activation

NOD1 activation occurs when host cells are subjected to the stimulating ligand, *meso*DAP from peptidoglycan. Cell line Hek-blue hNod1 (Invivogen, USA) can be used to show the activation of NOD1 when infected with bacterial species [251]. This cell line is co-transfected with the human NOD1 gene and an optimised secreted embryonic alkaline phosphatase (SEAP) reporter gene in Hek293 kidney cells. Once NOD1 is stimulated, NF- κ B and AP-1 binding sites are activated, inducing the production of SEAP which can then be detected in host cell media using spectrophotometry in real time.

Hek-blue hNOD1 cells were infected with heat inactivated *Rickettsia*, *Anaplasma*, *Wolbachia*, *Erlichia* and *Orientia*. Bacteria were rendered inactive after being boiled at 95°C for 30 minutes. Equivalent numbers of bacteria from each species were added to Hek-blue hNOD1 cells seeded in a 96 well plate. After 24hrs of infection, culture media was removed and replaced with SEAP detection media. The intensity of SEAP was quantified at 640nm using a spectrophotometer. *E. coli* was used as a positive control as well as γ -D-Glu-mDAP (iE-DAP) dipeptide. Statistical t-test analysis was achieved by comparing all species to *E. coli*.

Results presented higher absorbance intensity of SEAP for species with classic or intermediate peptidoglycan status compared to species with no peptidoglycan (**Figure 32**). These results verify the peptidoglycan predicted results by bioinformatics.

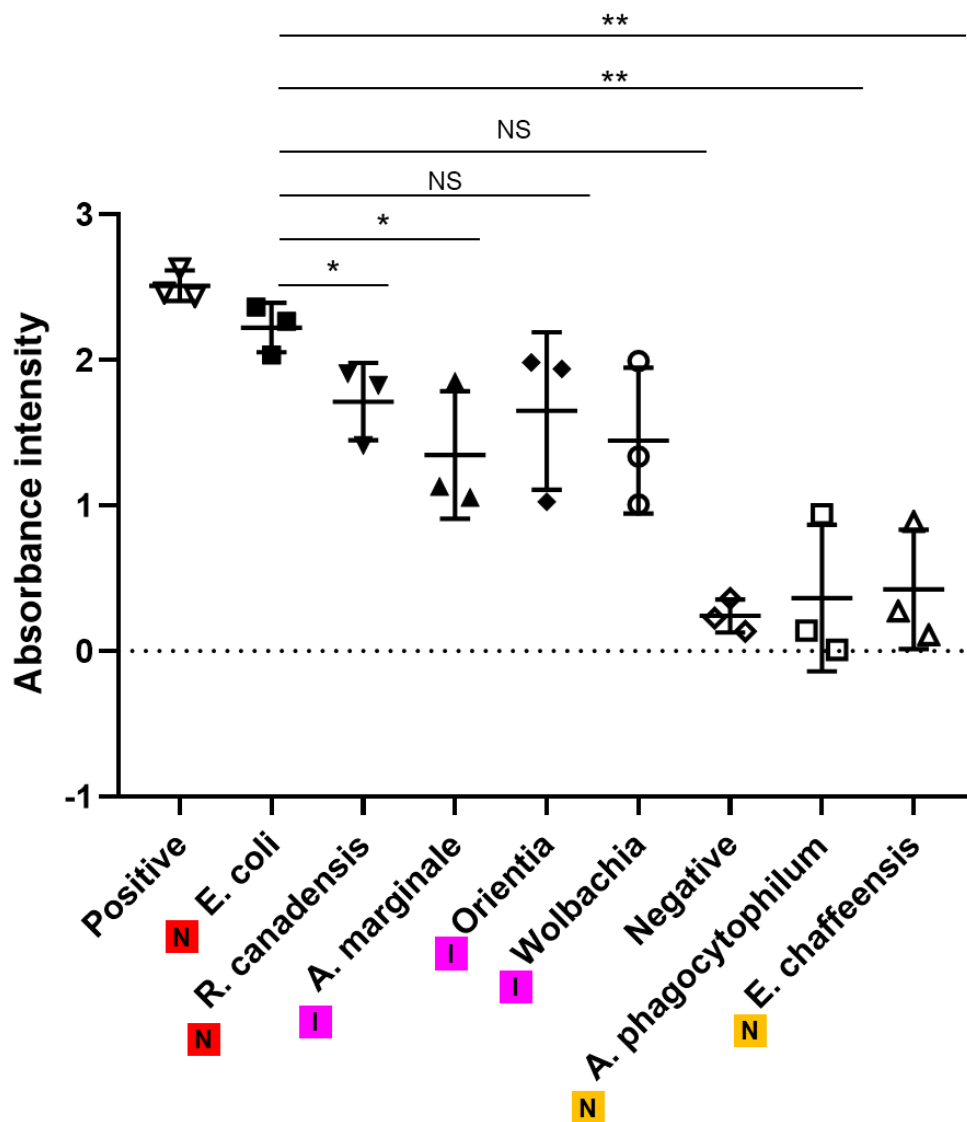


Figure 32. Nod1 activation in HEK293 cells infected with Rickettsiales species

Graph showing NOD1 activation by secreted embryonic alkaline phosphatase (SEAP) detection using spectrophotometry at the optical density of 640nm. Bacteria were added to transfected HEK293 cells and 3 days post infection, detection media was added to cells to allow SEAP detection. All detections were carried out in triplicate. A T-test was performed to compare conditions to *E. coli* SEAP detection and P values are illustrated as follows: ns ($P > 0.05$); * ($P \leq 0.05$); ** ($P \leq 0.01$).

Positive control = γ -D-Glu-mDAP (iE-DAP) dipeptide recognised by NOD1.

Negative control = cell culture media

C = Classical peptidoglycan, I = Intermediate peptidoglycan, N = No peptidoglycan

5.3.e Drug sensitivity

Drugs targeting the cell wall structure was used to show the effect on bacterial growth and structure. Bacteria were grown in various cells lines, seeded on 96 well plates. After one hour of infection, drugs were added for the duration of the infection cycle. After 4 days, bacterial quantification was carried out by real-time qPCR of 16s (**Figure 33**)

Drugs used in this experiment were chosen after the effect shown on *Orientia* in the previous chapter. These drugs were chloramphenicol, d-cycloserine, phosphomycin and penicillin. Chloramphenicol was the positive control in this experiment as it is known to be highly effective against Rickettsiales, targeting the 50S ribosome. D-cycloserine targets Ddl and Alr, phosphomycin targets MurA and penicillin targets DD-transpeptidases. Results for *R. canadensis* show that as expected, bacteria did not grow in the presence of cell-wall targeting drugs. *A. marginale* was sensitive to penicillin and D-cycloserine, suggesting the presence of a peptidoglycan-like structure that is required for viability, but not phosphomycin. The reason for this insensitivity is unknown, since *A. marginale* encodes *murA*. *E. chaffeensis* was not affected by any of the cell wall targeting drugs as expected since this species has no genes for peptidoglycan synthesis. Given the slow growth of *Wolbachia* and *A. phagocytophilum* these assays could not be performed on those species.

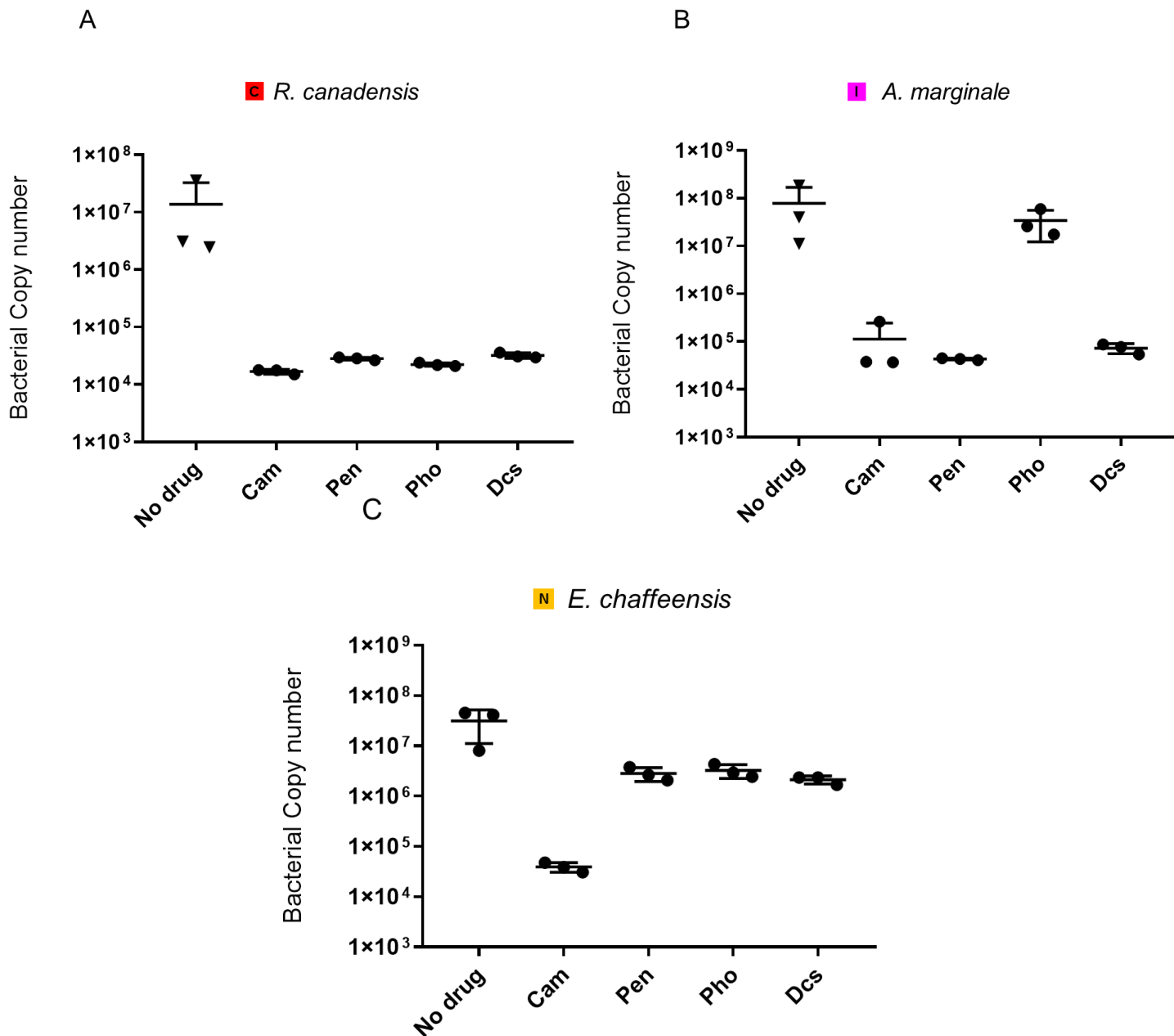


Figure 33. The effect of cell wall targeting drugs on the growth of Rickettsiales species

A-C: Bacterial copy number per well of a 96 well plate, 4 days post infection in the presence of various drugs. Each condition was carried out in triplicate. The copy number of 16s was measured by RT-qPCR.

Pen = Penicillin, targets PBPs

Pho = Phosphomycin, targets MurA

Dcs = D-cycloserine, targets Ddl and Alr

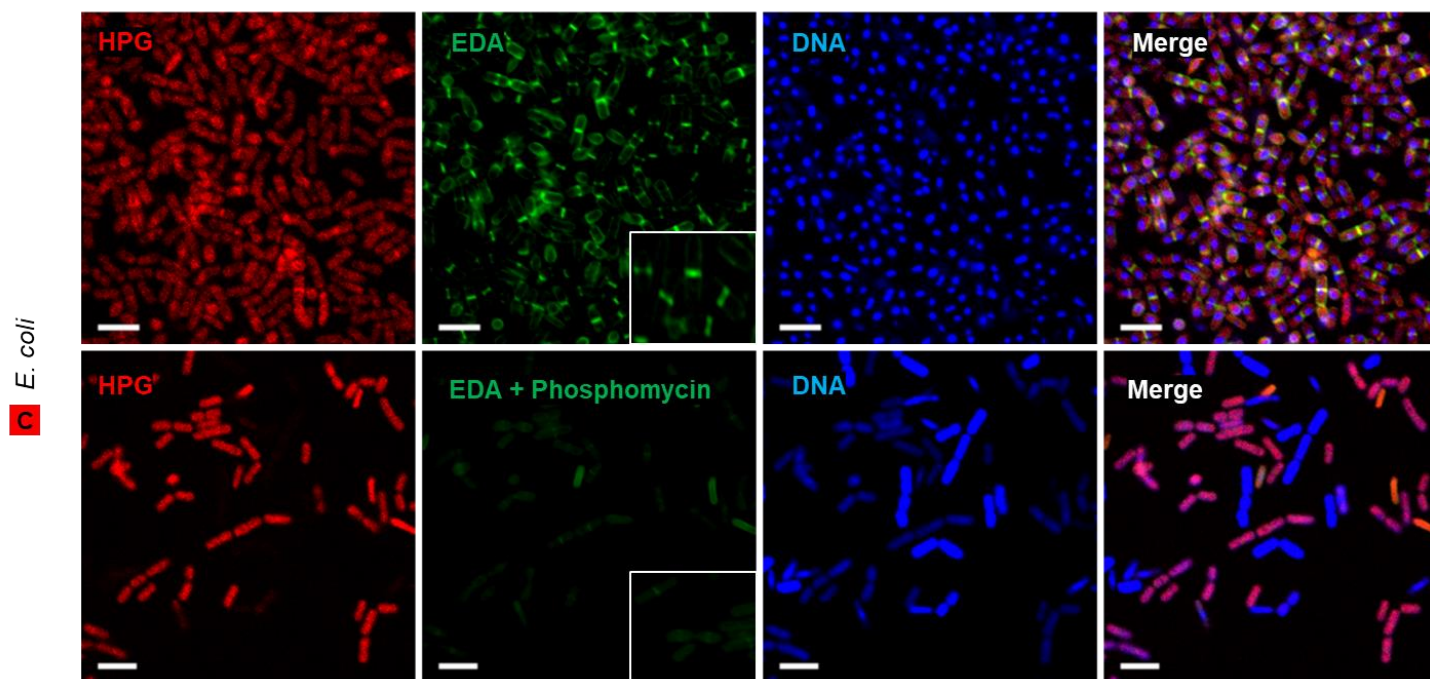
Cam = Chloramphenicol, targets 50s

C = Classical peptidoglycan, I = Intermediate peptidoglycan, N = No peptidoglycan

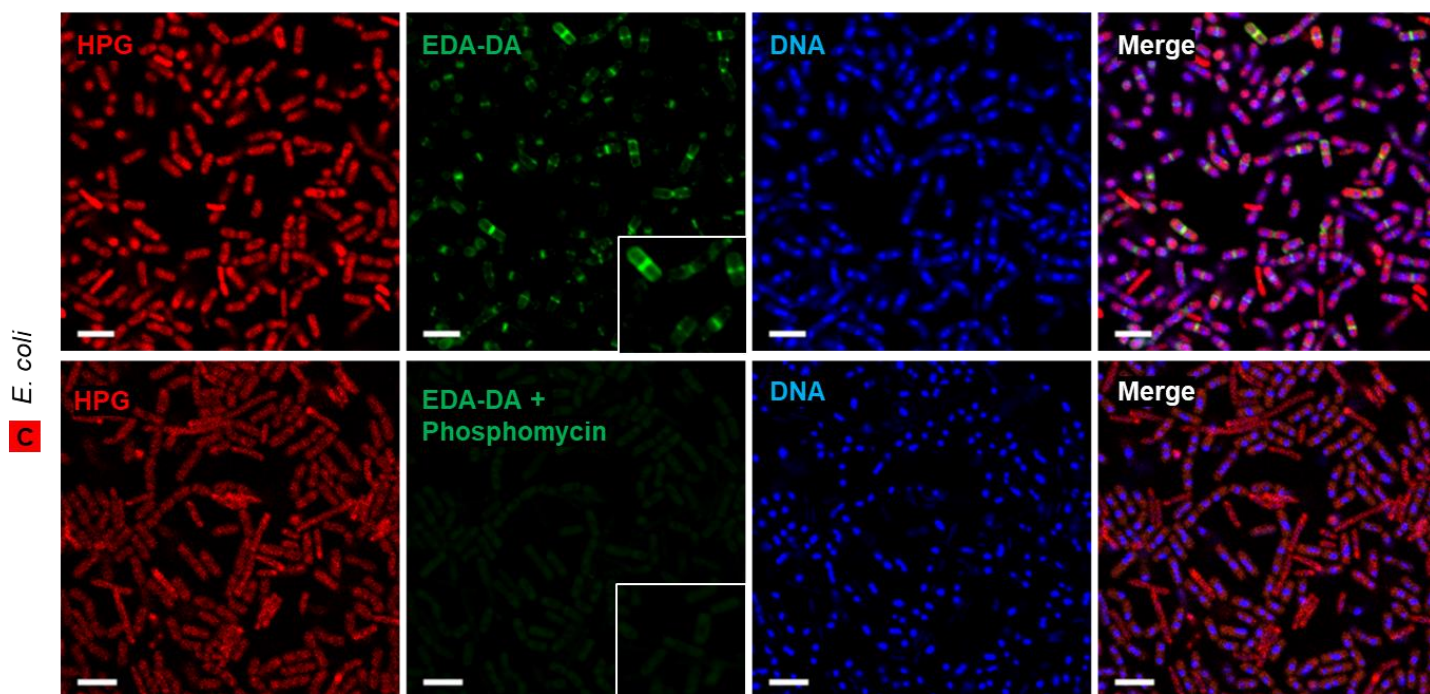
5.3.f EDA and EDA-DA

The presence of peptidoglycan can be shown by the incorporation of EDA and EDA-DA probes, as shown in the case of *Orientia*. EDA and EDA-DA were first tested in *E. coli* and *B. subtilis* to verify labelling protocols (**Figure 34**). Peptidoglycan labelling was presented at the septum of the bacteria but in some cases only in the cell wall. Due to the similarity in labelling, only EDA was used for peptidoglycan labelling in Rickettsiales species in this chapter.


A

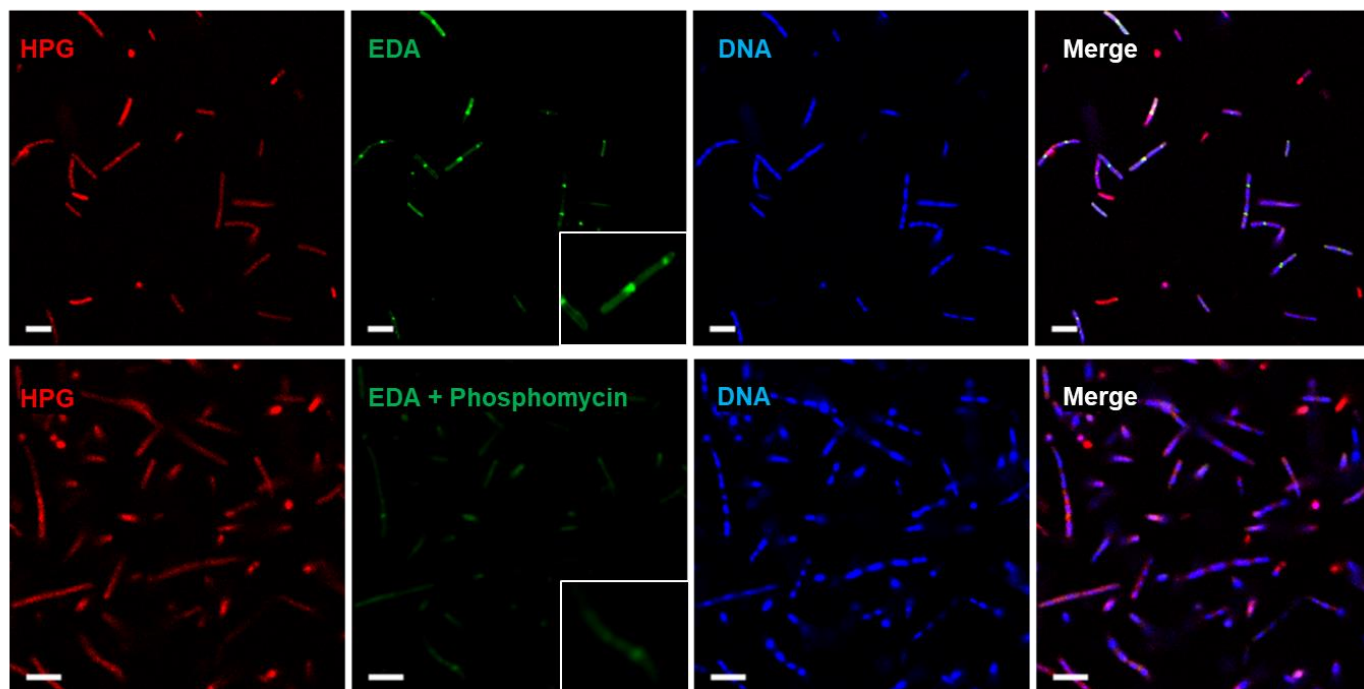


B




C

 *B. subtilis*



D

 *B. subtilis*

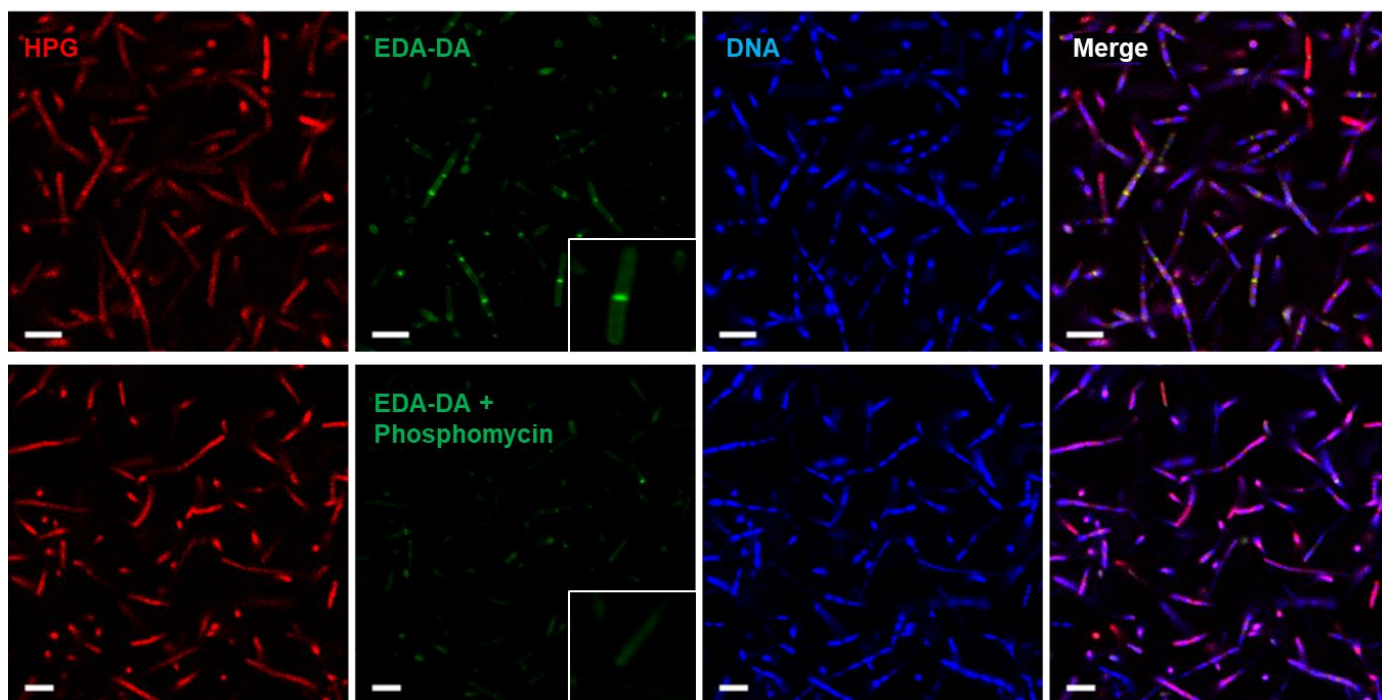


Figure 34. Fluorescence microscopy of peptidoglycan probes in *E. coli* and *B. subtilis*

A-B: EDA and EDA-DA probes were added to *E. coli* with HPG for 10 minutes during exponential phase of growth.

C-D: EDA and EDA-DA probes were added to *B. subtilis* with HPG for 10 minutes during exponential phase of growth.

Both probes presented septal labelling.

Click chemistry was carried out for both label

Scale bar = 5µm

C = Classical peptidoglycan

Phosphomycin inhibits MurA activity in peptidoglycan synthesis. EDA was added to infected cells at 1mM for different incubation times for each species without or without phosphomycin in order to test the specificity of labelling. After the addition of a methionine probe, which was conjugated to an alkyne moiety in order to enable double click labelling, cells were fixed, click-labelled and imaged. *R. canadensis*, which encodes all peptidoglycan genes, presented the clearest peptidoglycan labelling (**Figure 35**) which occurred most frequently at the septum of the bacteria. With the addition of phosphomycin, bacteria became rounder and many had lysed, indicated by the lack of metabolically active methionine-labelled *R. canadensis*. Most important, EDA was not present with the addition of phosphomycin.

Intermediate status bacteria, *A. marginale* and *Wolbachia* presented very similar results to *Orientia*, another intermediate bacterial species. Both bacterial species exhibited uniform peptidoglycan labelling of the whole bacteria with no localised cell wall or septum labelling (**Figure 36, Figure 37**). The high background of EDA throughout the host cell cytoplasm was again visible, as seen after EDA *Orientia* labelling (**Figure 24**). It is interesting that only the intermediate bacterial species show this high background. This high background was not observed in uninfected cells and therefore was not a labelling artefact. As with *Orientia* the high background may be due to peptidoglycan that has been shed into host cells (**Figure 40**). With the addition of phosphomycin, EDA labelling was still partly visible even though the bacterial structure had clearly become affected by the drug. The residual EDA labelling with phosphomycin may be explained by the slower turnover of peptidoglycan synthesis so it would take longer for incorporated EDA to be replaced.

In bacterial species with no peptidoglycan, *A. phagocytophilum* and *E. chaffeensis*, show no EDA label at all and no EDA background labelling in host cells (**Figure 38, Figure 39**). Bacterial cell shape was unaffected by the addition of cell wall targeting drugs. This verifies that these species do not synthesise peptidoglycan.

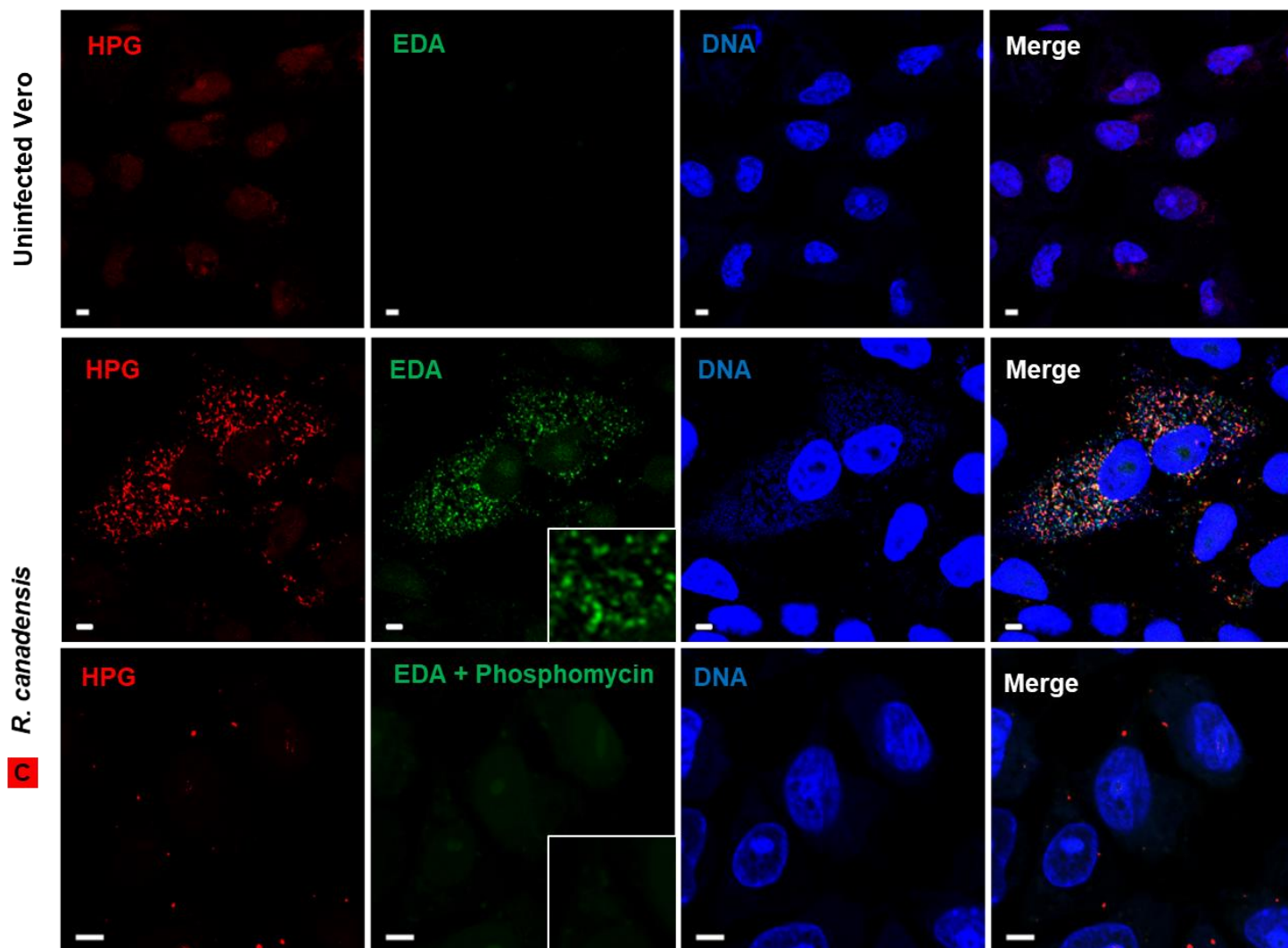


Figure 35. Peptidoglycan labelling in *R. canadensis*

EDA was added to infected vero cells at day 3 with HPG and fixed after 30 minutes of probe incubation. EDA labelling presented septum labelling in bacteria.

Phosphomycin targets MurA.

Scale bar = 5µm

C = Classical peptidoglycan

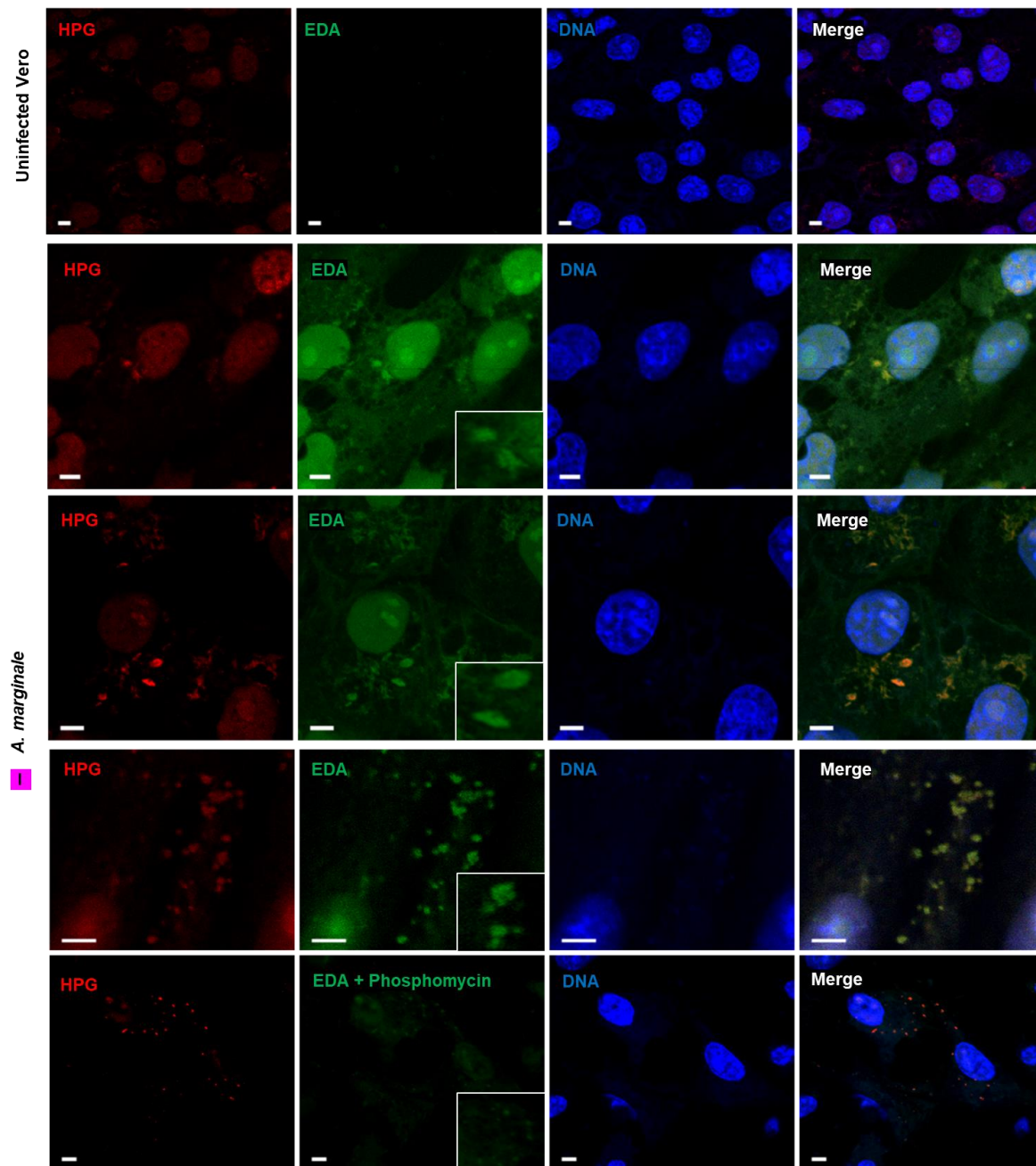


Figure 36. Peptidoglycan labelling in *A. marginale*

EDA was added to infected vero cells at day 3 with HPG and fixed after 30 minutes of probe incubation. EDA labelling presented surface labelling in bacteria.

Phosphomycin targets MurA.

Scale bar = 5µm

I = Intermediate peptidoglycan,

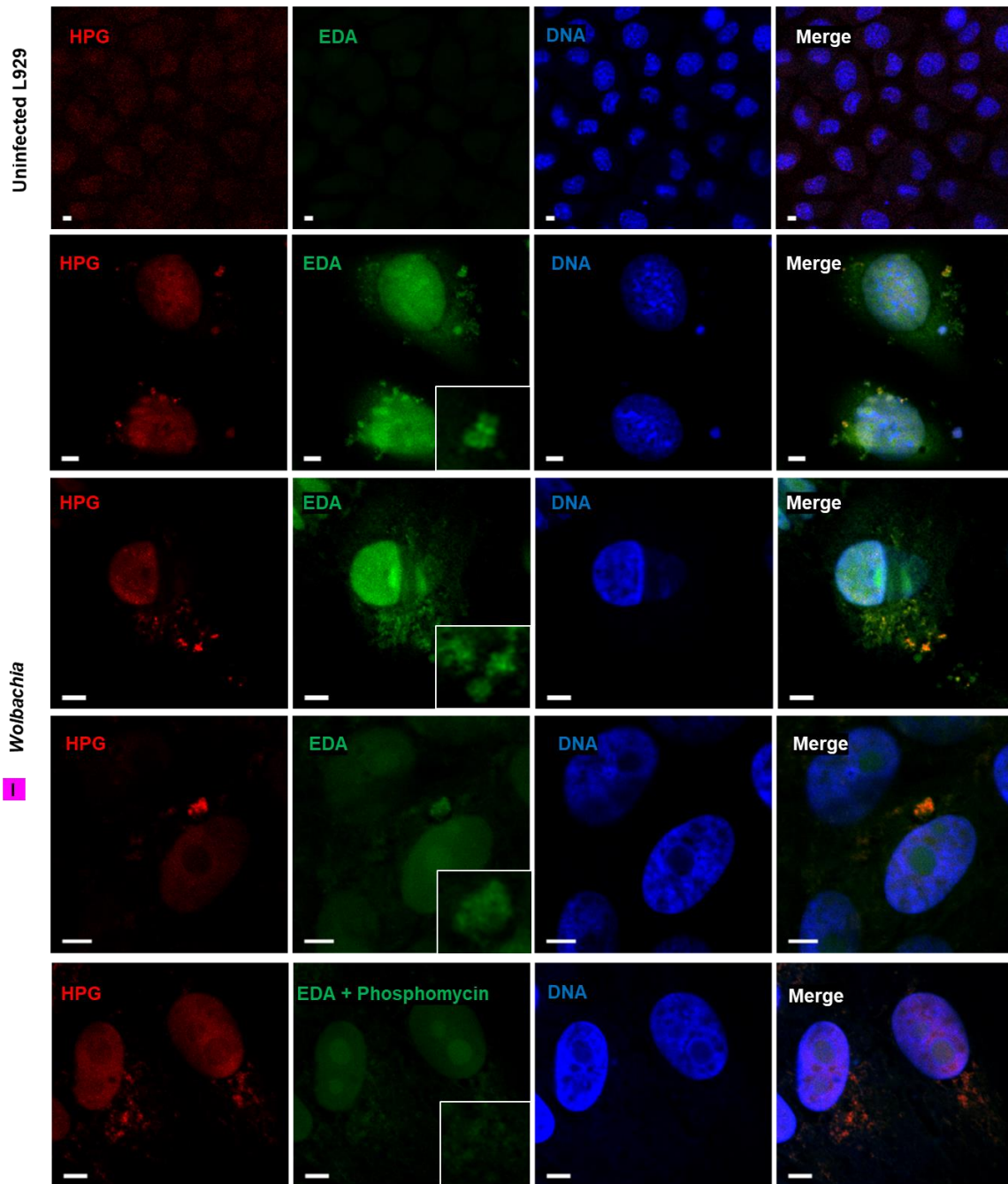


Figure 37. Peptidoglycan labelling in *Wolbachia*

EDA was added to infected L929 cells at day 3 with HPG and fixed after 4 hours of probe incubation. EDA labelling presented surface labelling in bacteria.

Phosphomycin targets MurA.

Scale bar = 5µm

I = Intermediate peptidoglycan

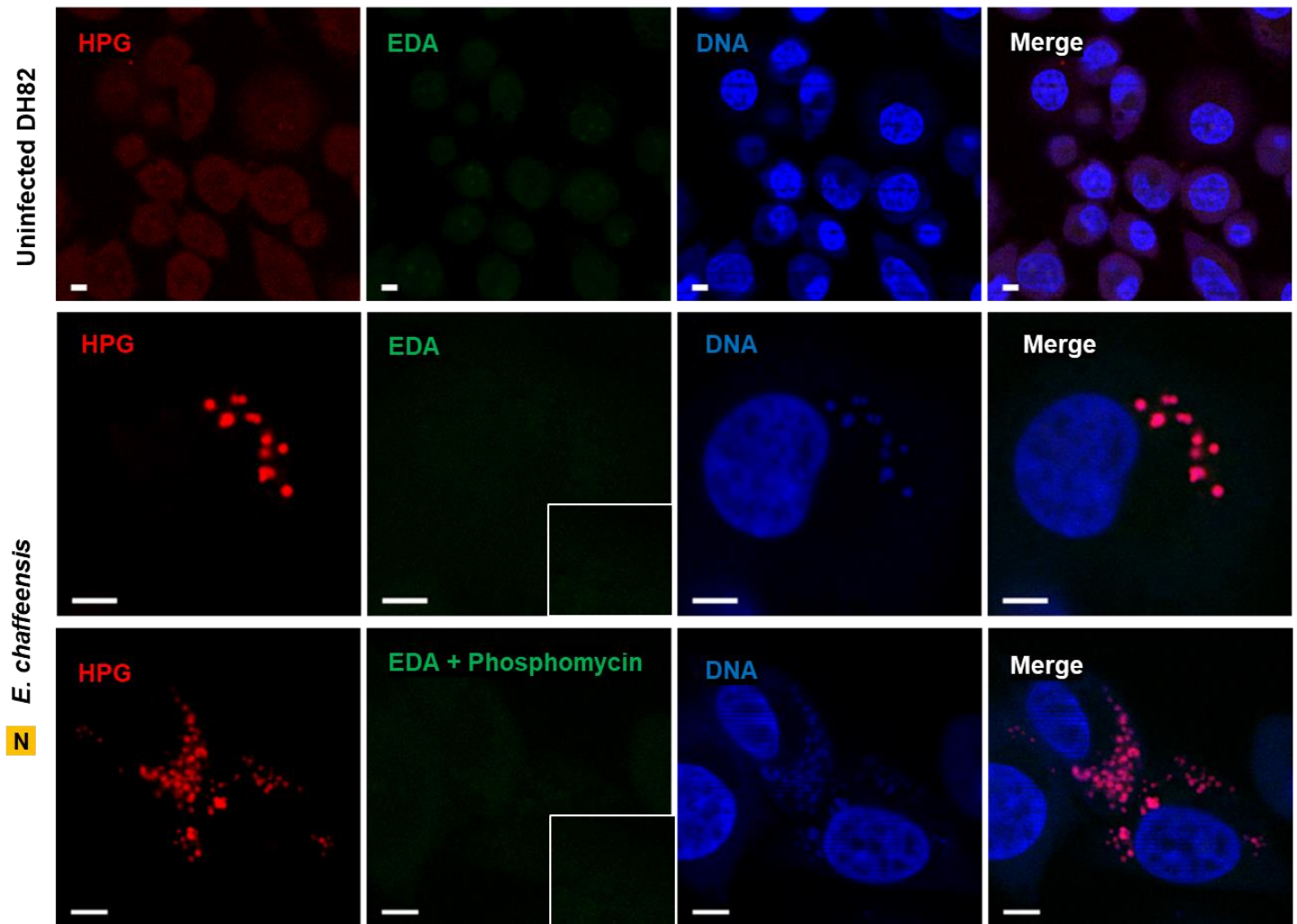


Figure 38. Peptidoglycan labelling in *E. chaffeensis*

EDA was added to infected DH82 cells at day 3 with HPG and fixed after 30 minutes of probe incubation. EDA labelling was absent in bacteria.

Phosphomycin targets MurA.

Scale bar = 5µm

N = No peptidoglycan

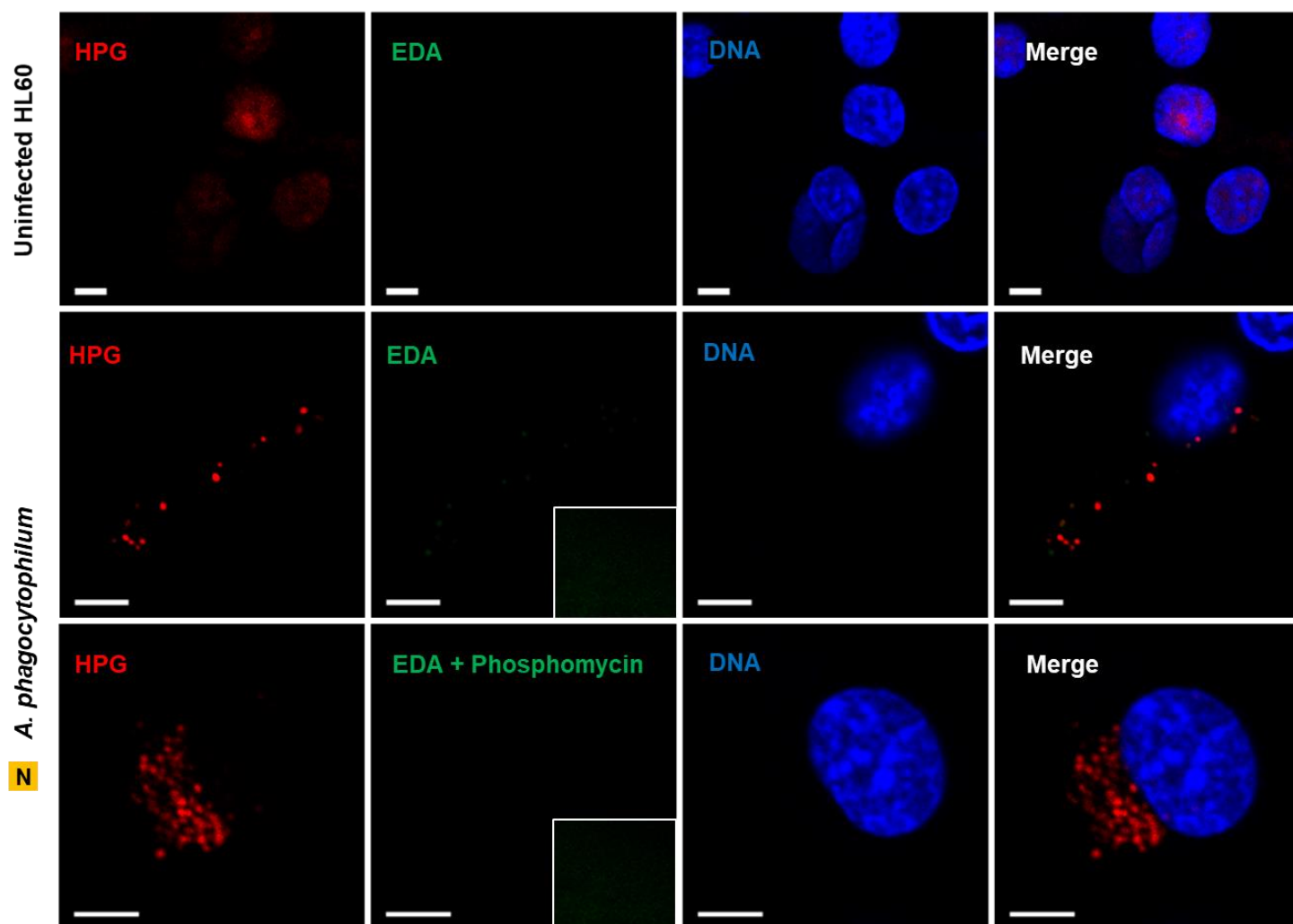


Figure 39. Peptidoglycan labelling in *A. phagocytophilum*

EDA was added to infected HL60 cells at day 3 with HPG and fixed after 30 minutes of probe incubation. EDA labelling was absent in bacteria.

Phosphomycin targets MurA.

Scale bar = 5µm

N = No peptidoglycan

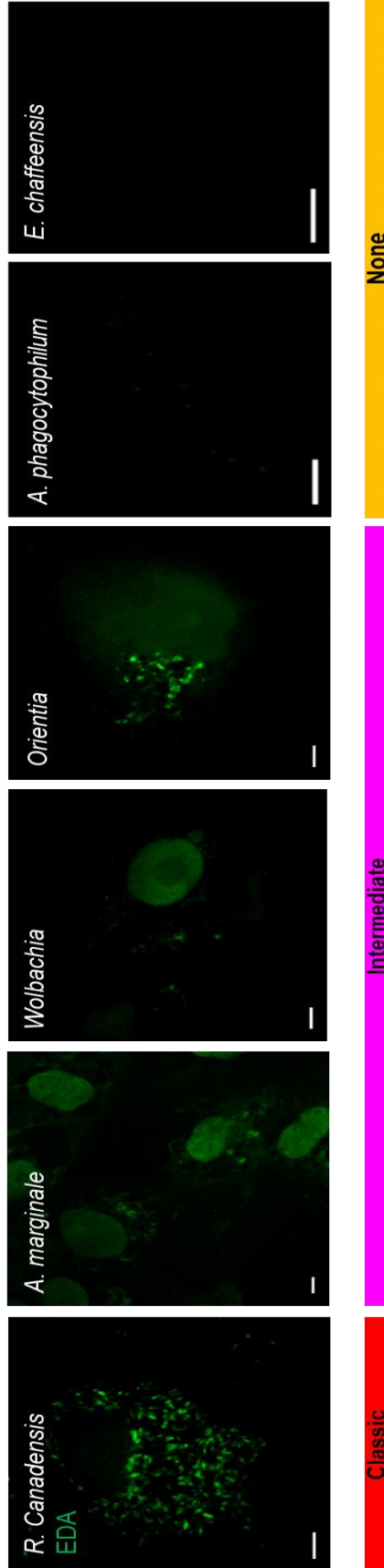


Figure 40. A summary of EDA labelling in all Rickettsiales species researched in this thesis

Rickettsiales species can be identified as one of 3 groups based on peptidoglycan present in the cell wall.

Scale bar = 5µm

5.3.g Cell wall sensitivity to osmotic stress

Peptidoglycan in the cell wall provides osmotic protection. Without it, bacteria may be vulnerable to lysis in environments of low osmolarity. To investigate the structural rigidity of Rickettsiales species, bacteria were treated in conditions that would exert different osmotic pressure on cells. Isolated *R. canadensis*, *A. marginale* and *A. phagocytophilum* were resuspended in culture media, phosphate buffered saline (PBS), water and sucrose phosphate glutamate (SPG) (**Figure 41**).

These buffers for bacterial treatment were chosen according to the osmotic pressure they may cause. For instance, SPG and culture media are isotonic so will not disrupt osmotic pressure in the bacteria. On the other hand, water is hypotonic and osmotic flow will cause the pressure inside the bacteria to be elevated and cause osmotic lysis. Water can freely move across the cell membrane of bacteria so that there is risk of high osmotic pressure. To withstand turgor pressure, the plasma membrane is protected by peptidoglycan, so the absence of it can cause lysis of the cell.

After 10 minutes of exposure to different buffers bacteria were pelleted and then imaged by confocal microscopy after methionine labelling. *R. canadensis* maintained its structure in media and SPG as expected but in water bacteria became rounder (**Figure 42**). Bacteria did not appear to lyse completely which indicates a strong cell wall present. *A. marginale* also maintained structural composure in SPG and media and in water became rounder but did not lyse. In contrast, *A. phagocytophilum* appeared to lyse completely in water compared to SPG and media conditions. This would be expected as *A. phagocytophilum* does not consist of any genes for peptidoglycan synthesis so its cell wall must be structurally vulnerable compared to the other two species. Taken together this indicates that both *R. canadensis* and *A. marginale* have a structure conferring osmotic protection whilst *A. phagocytophilum* does not, further supporting the hypothesis of the presence of peptidoglycan-like structures in the first two and not the latter.

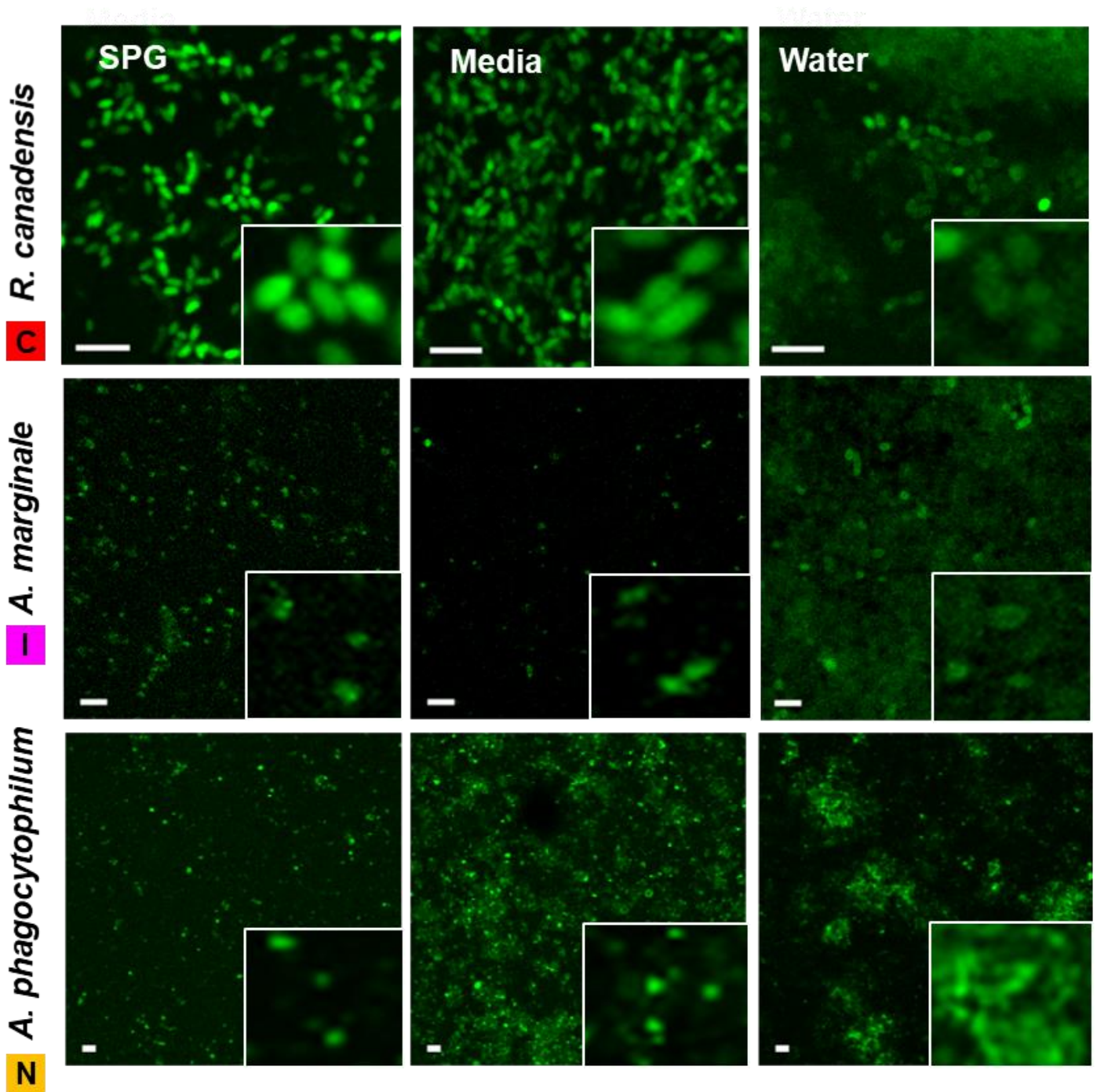


Figure 41 . Fluorescence microscopy sensitivity to osmotic pressure of Rickettsiales species

Bacteria were treated with SPG, culture media and water for 10 minutes with the addition of a methionine probe.

Bacteria were then fixed on to glass slides for click chemistry labelling and imaged.

Scale bar = 5µm

C = Classical peptidoglycan, I = Intermediate peptidoglycan, N = No peptidoglycan

5.3.h. Cell wall sensitivity to lysozyme and mutanolysin

Intermediate peptidoglycan species do not have class A PBPs, so are predicted to have no glycosyltransferase activity. Lysozyme and mutanolysin both target the β 1,4 linkage, which is catalysed by glycosyltransferase, between lipid II precursors. *Orientia* presented a damaged cell wall structure after being when treated with lysozyme, confirming the presence of the polymerisation of β 1,4 linkage between glycans despite an absence of class A PBPs. To investigate whether Rickettsiales species have a peptidoglycan structure polymerised by glycosyltransferase activity, bacteria were treated with SPG with 5ug/ml lysozyme and 5ug/ml mutanolysin for 10 minutes (**Figure 42**). Following this, bacteria were pelleted and then imaged by confocal microscopy after methionine labelling

After lysozyme and mutanolysin treatment, *R. canadensis* became damaged and many bacteria had lysed as shown by the surrounding remnants. *A. marginale*, is missing class A PBPs but still become lysed by lysozyme treatment. Bacteria also became lysed after treatment with mutanolysin and many bacteria increased in size. This suggests that due to the action of the drugs on the glycosidic bond between residues, peptidoglycan is degraded and so without a strong cell wall, the bacteria becomes more vulnerable to osmotic stress. However, *A. phagocytophilum* did not change morphology in lysozyme or mutanolysin, which is further evidence it does not have a peptidoglycan structure.

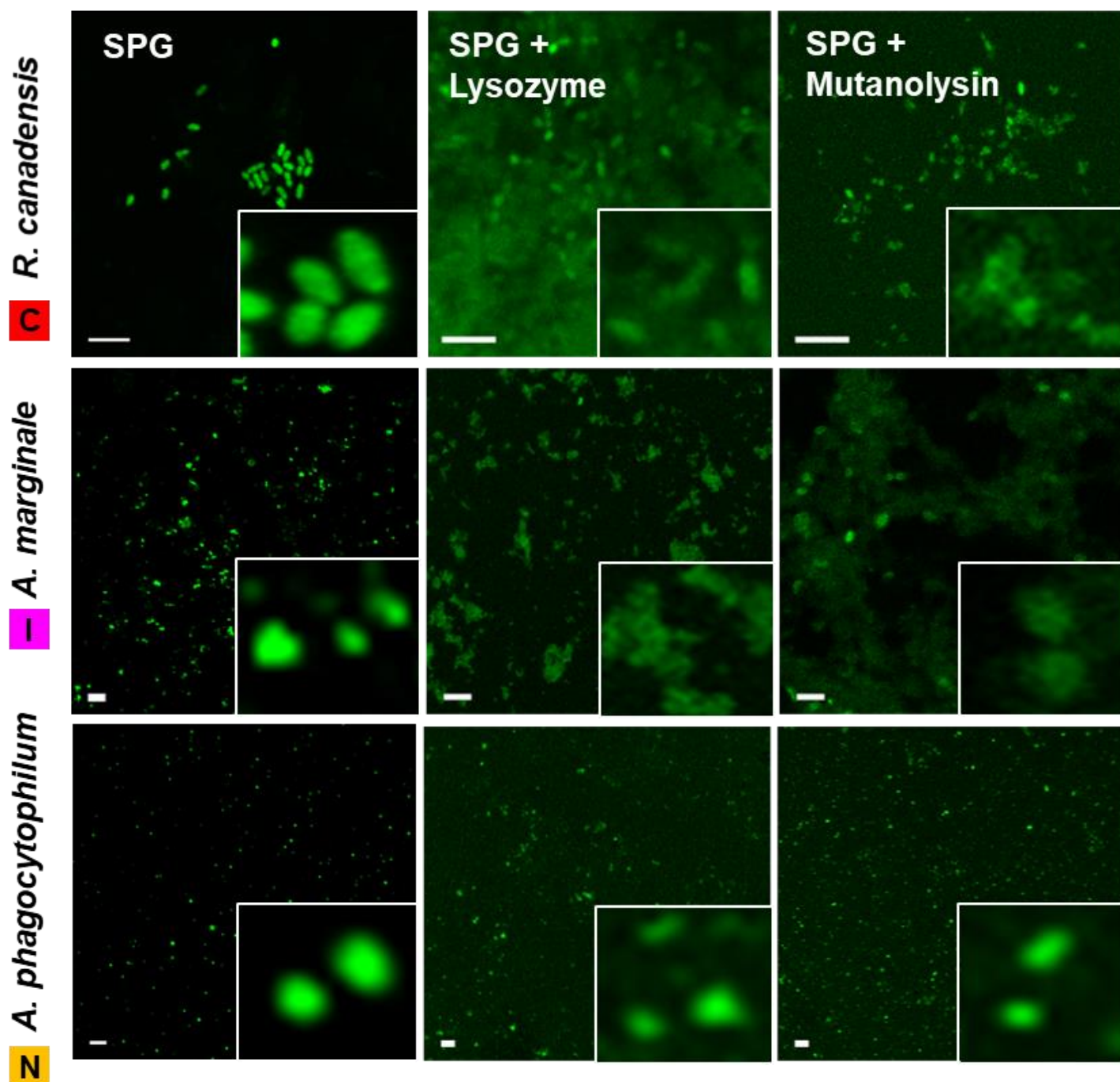


Figure 42. Fluorescence microscopy cell wall sensitivity to lysozyme and mutanolysin in Rickettsiales species

Bacteria were treated with SPG with and without 5ug/ml lysozyme and mutanolysin for 10 minutes with the addition of a methionine probe. Bacteria were then fixed on to glass slides for click chemistry labelling and imaged.

Scale bar = 5µm

C = Classical peptidoglycan, I = Intermediate peptidoglycan, N = No peptidoglycan

5.3.i Heterogeneity in peptidoglycan probe labelling

As all species in this thesis have different life cycles, the rate of peptidoglycan synthesis will also occur at various times of each infection cycle. This complicated labelling as the optimal bacterial growth state was needed for labelling.

Probes EDA and EDA-DA were used during this research project with Rickettsiales species. The mechanism of incorporation of these probes is not fully known so there was discussion of how these probes may label bacteria differently. However, with both EDA and EDA-DA labelling, differences were noted in localisation of the probe. In some cases, peptidoglycan labelling was consistently at the septum of the bacteria but in other cases peptidoglycan was labelled only at the cell wall (**Figure 43**). This variation of labelling of the same species indicates that peptidoglycan labelling will vary according to the bacterial state inside host cells.

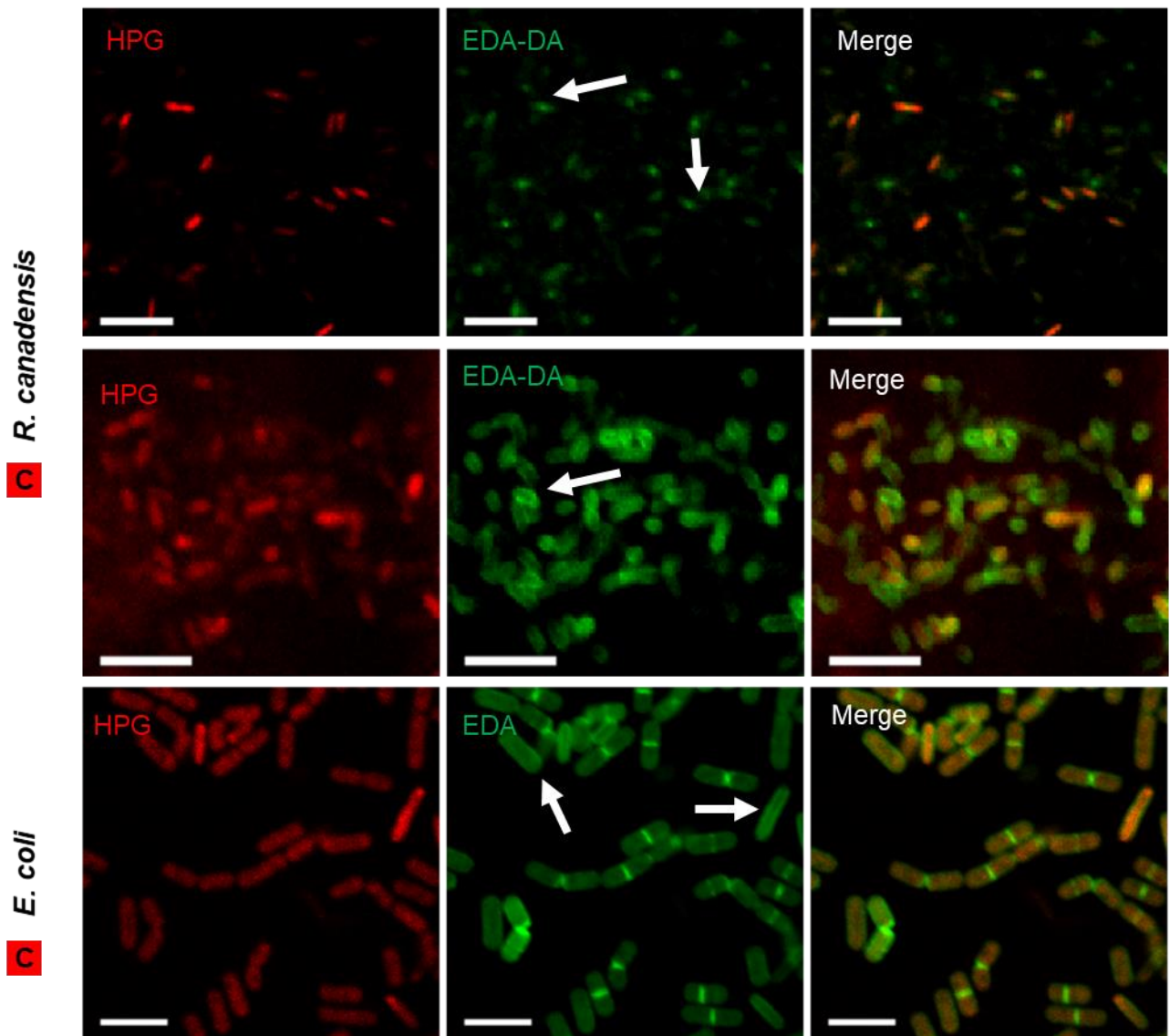


Figure 43. Heterogeneity in EDA/EDA-DA peptidoglycan labelling

Both rows show infection in vero cells at day 3 infection. The top row presents EDA-DA labelling as only localised to the septum.

However, the bottom row shows EDA-DA labelling the cell wall of many of the bacteria.

Scale bar = 5μm

C = Classical peptidoglycan

5.4. Discussion

Peptidoglycan in Rickettsiales species had not yet been identified and characterised. With the advent of new peptidoglycan labelling probes and with the bioinformatics prediction of a new peptidoglycan intermediate group, the presence or absence of peptidoglycan was tested for in a group of Rickettsiales species. *Orientia* was thought to not synthesise peptidoglycan but with most genes present to do so, which was showed in the last chapter of this thesis that it does have a peptidoglycan-like structure in its cell wall. Bioinformatics predicted that Rickettsiales could be classified into three groups, those with classical peptidoglycan (*R. canadensis*) those with intermediate peptidoglycan (*Wolbachia*, *A. marginale*, *O. tsutsugamushi*) and those with no peptidoglycan (*A. phagocytophilum*, *E. chaffeensis*). In this chapter, I used a combination of approaches to test this hypothesis.

First, protocols were developed for the growth of species in host cell lines as they were not previously grown in the lab. Also, without antibodies available, labelling species was critical. All Rickettsiales species successfully labelled with clickable methionine probes and RNA FISH for 16s. This labelling allows a counter-label for peptidoglycan EDA labelling.

I found that intermediate status species *A. marginale* and *Wolbachia* are sensitive to cell wall targeting drugs, activate the peptidoglycan binding host cell protein NOD1 and can be labelled with the D-alanine probe EDA (and EDA-DA). The levels of NOD1 activation were similar to that for *R. canadensis*, which is expected to have higher levels of peptidoglycan. This might reflect a limitation of the dynamic range of this assay.

Similar to what was found with *Orientia* in the previous chapter, EDA labelling in the intermediate species proved to be less efficient compared to *R. canadensis* and generated high levels of fluorescent background in host cells (**Figure 40**). This suggests that these species have lower levels of peptidoglycan. Since it is not known how exactly EDA is integrated, it may also be that

the probe can only integrate in specific stages of peptidoglycan synthesis (such as during peptide chain formation). If synthesis is slower or paused during the Anaplasmataceae infection cycle labelling would therefore be reduced compared to classical status species. It is known that these species exist in two morphologically different states in the host cell and are enclosed in a vacuole for much of the infection cycle. The extracellular infectious dense core cells must be structurally different in the cell wall compared to the reticulate cells which are enclosed in the vacuole.

Another explanation for the low levels of labelling and high host cell background for EDA, is that the bacteria may be shedding peptidoglycan. At times, it may be more beneficial for these intermediate bacteria to have no peptidoglycan in the cell wall. Some bacterial species with established peptidoglycan structures are capable of inducing L-form phase, a cell wall-free state [252]. In L-form, bacteria become osmotically sensitive but cell wall targeting antibiotic resistant [167]. Division can still occur, not by the usual FtsZ machinery, but by unregulated blebbing. If *Orientia* or *A. marginale* were able to induce L-form status during their infection cycles, the bacteria may remodel their cell wall by down regulating genes required for peptidoglycan synthesis. This could cause the breakdown and emission of peptidoglycan fragments into the host cell environment which may explain the very high background visualised after EDA peptidoglycan labelling in only intermediate peptidoglycan species. However, the peptidoglycan that is shed is likely to trigger the innate immune response through activation of NOD1 and other receptors.

Like *Orientia*, these intermediate species have a peptidoglycan-like structure present in their cell wall but lack Class A PBP genes which are classically known to be responsible for glycosyltransferase activity. I found that these organisms are sensitive to lysozyme which cleaves glycan chains, indicating the presence of a polymerized peptidoglycan-like structure. This suggests that in the absence of Class A PBPs or monofunctional glycosyltransferases these species possess alternate pathways for joining the lipid II precursors. This may occur through the

coordination of other complexes such as the SEDS glycosyltransferase proteins, FtsW and RodA [181].

Species with almost no genes for peptidoglycan biosynthesis, *A. phagocytophilum* and *Ehrlichia chaffeensis* did not present any evidence of peptidoglycan synthesis by drug sensitivity, NOD1 activation or D-alanine labelling. Without a peptidoglycan cell wall, both proved to be structurally vulnerable when lysed from host cells. With reduced or no peptidoglycan in the cell wall, how do bacteria protect themselves from osmotic pressure? Mycoplasma are cell wall deficient and have adapted to this state over millions of years of evolution. Instead of peptidoglycan, the cell membrane is composed of sterols which are needed for its growth [211] [212] and contribute to the durability of the cell membrane against osmotic shock [213]. Instead of a peptidoglycan structure, *A. phagocytophilum* and *Ehrlichia* may acquire protection by cholesterol integration [139]. Without a peptidoglycan-like structure, the dense core and reticulate cells may instead have a crosslinked membrane protein structure to prepare to their external environment and host interactions, much like *Chlamydia* [253] and *Orientia*.

Overall Conclusions

***Orientia* can be fluorescently labelled for live and fixed imaging**

Orientia tsutsugamushi is a neglected pathogen and its cell biology is poorly understood. In this work I have expanded the toolkit available for working with this bacterium in the laboratory. I have shown that *Orientia* can be fluorescently labelled by antibodies against major membrane surface proteins, dyes which label amino acids, DNA or membrane structures and chemical probes for methionine, RNA and peptidoglycan labelling. This toolkit of chemical probes is likely to be useful for working with other genetically intractable obligate intracellular bacteria including other Rickettsiales.

***Orientia* has a peptidoglycan-like structure in its cell wall**

Using mass spectrometry of *meso*DAP, gene expression analysis, drug sensitivity assays, peptidoglycan probe integration and host cell NOD1 activation, I have shown that *Orientia* does synthesise a peptidoglycan-like structure that is vital for its viability. I found that this structure is further supported by a disulphide crosslinked membrane protein network in the bacterial outer membrane.

Rickettsiales species can be classified into three groups: classic peptidoglycan, intermediate peptidoglycan and peptidoglycan negative

Bioinformatics analysis led to the prediction that Rickettsiales could be classified into three groups based on their peptidoglycan status: classic, intermediate and negative. Here I have used peptidoglycan binding host cell protein NOD1 to show which species are able to cause its activation. *R. canadensis*, *A. marginale*, *Orientia* and *Wolbachia* were able to stimulate NOD1

whilst *A. phagocytophilum* and *Ehrlichia* were not. Furthermore, *R. canadensis*, *A. marginale*, *Orientia* and *Wolbachia* proved to be sensitive to most cell wall targeting drugs. Cell wall targeting drugs did not affect the growth of *A. phagocytophilum* and *Ehrlichia*. D-alanine peptidoglycan label EDA successfully labelled peptidoglycan in *R. canadensis*. EDA labelling occurred in *A. marginale*, *Orientia* and *Wolbachia* but was not consistent and sometimes unspecific, generating host labelling. No EDA was visible in *A. phagocytophilum* or *Ehrlichia*. Additionally, *R. canadensis* and *A. marginale* showed sensitivity to glycosyltransferase inhibitors whereas *A. phagocytophilum* did not.

R. canadensis, *A. marginale* and *A. phagocytophilum* maintained structural integrity in media and SPG. *R. canadensis* and *A. marginale* showed evidence of osmotic pressure in water. On the contrary, *A. phagocytophilum* did not bear well under osmotic pressure in water and lysed.

Taken together, these results confirm the bioinformatics-based prediction that *R. canadensis* synthesises classic peptidoglycan *A. marginale*, *Orientia* and *Wolbachia* synthesise an intermediate peptidoglycan-like structure and *A. phagocytophilum* and *Ehrlichia* do not synthesise a peptidoglycan cell wall.

Future Directions

This research leads to further questions.

The development of a toolkit for labelling *Orientia* and other genetically intractable obligate intracellular bacteria opens a number of new research avenues. For example, live imaging of *Orientia* is a key new tool that will allow us to understand its infection cycle in the host cell. Specifically, using the new live imaging probes I observed that *Orientia* forms large aggregates that can enter host cells as a collective. Future work will focus on determining the mechanism of entry of these aggregates. Also, *Orientia* is known to colocalise to the microtubules of the host cell for transport. By live imaging and using fluorescent dyes, the colocalisation of bacteria and dynein proteins may be visualised in real time. One key step in the life cycle of *Orientia*, is the movement from the perinuclear region to the cell periphery for bacterial exit. By using fluorescent labelling, the host factors involved in this transit can be identified as well as the key proteins *Orientia* may utilise.

Using the new antibodies generated here we discovered that not all subpopulations of bacteria could be labelled with all antibodies. This led to a new research avenue in which the distinct stages of the intracellular infection cycle of *Orientia* are being defined. This work is using both immunofluorescence and RNA FISH labelling to look at heterogeneity in gene expression.

In this thesis I showed that a group of Rickettsiales bacteria including *Orientia tsutsugamushi*, *Anaplasma marginale* and *Wolbachia* synthesise an intermediate peptidoglycan-like structure. Further research needs to be completed in order to understand exactly when peptidoglycan is synthesised during the infection cycle for Rickettsiales species. As some species exist in dense and reticulate cell form, the level of peptidoglycan in these states must be investigated especially as the bacteria are changing in size whilst remaining the same shape. There are still new techniques to be used which can further validate this work. EDA integration generated high

background in host cells. New probes have recently been developed which only become fluorescent once incorporated into transpeptidase reactions [254].

Though this research may help to understand the timing of peptidoglycan synthesis, the question remains by what mechanism Rickettsiales species make peptidoglycan. In other organisms the peptidoglycan complex is managed by cytoskeletal proteins FtsZ at the septum and MreB at the cell wall. FtsZ is vital for cell division. It would be interesting to image the Z-ring during division in Rickettsiales species and identify the colocalisation with peptidoglycan. Additionally, investigating whether peptidoglycan negative species such as *A. phagocytophilum* and *Ehrlichia* exhibit the Z-ring at the septum would indicate how these cells divide. The existence of FtsZ in bacteria and its role in division can be tested by an FtsZ inhibitor. Various mechanisms have been targeted to inhibit FtsZ such as a benzofuroquinolinium derivative which targets the GTPase and polymerisation activity [255, 256].

Could peptidoglycan positive species switch on and off peptidoglycan synthesis similar to L-forms described in free-living bacterial species? *Anaplasma marginale* for instance, exists in two forms during its infection cycle. It might be that one state down regulates peptidoglycan synthesis to allow prolonged survival inside host cells. L-form bacteria are known to divide without FtsZ [252] so this could be further investigated in intermediate peptidoglycan species by analysing the division of the bacteria and identifying the presence or absence of the Z-ring.

MreB and peptidoglycan work together during cell elongation as a unit. The localisation of MreB in Rickettsiales and the rate of peptidoglycan may provide insight to how and when the bacteria remodels its peptidoglycan. I spent time during my PhD working on MreB in *Shigella* and actin polymerization (not described in this thesis but published recently) [257]. MreB is known to drive actin polymerisation in *Shigella* [258] [257]. It is known that spotted fever group Rickettsial species can polymerise actin inside host cells [154]. The colocalisation of actin polymerisation and nascent peptidoglycan synthesis may indicate any relationships between the two.

Another key question is how the structure of peptidoglycan in intermediate peptidoglycan species is composed. Results from this thesis show that the linkage between the glycan residues is sensitive to lysozyme and mutanolysin, which both to hydrolyse β -1,4 linkages between MurNac and GlcNac residues. The absence of class A PBPs indicate that the glycosyltransferase activity must be performed by an alternate mechanism.

In summary, the research presented in my thesis opens several exiting new areas of research into the host pathogen cell biology and bacteriology of a medically important and understudied group of obligate intracellular bacteria.

Appendix 1

Abbreviations

CFSE = Carboxyfluorescein succinimidyl ester

CFDA-SE = carboxyfluorescein diacetate succinimidyl ester

HADA = HCC amino D-alanine

HALA = HCC amino L-alanine

EDA = Ethynyl-D-alanine

EDA-DA = Ethynyl-D-alanyl-D-alanine

GlcNac = N-acetyl glucosamine acid

MurNac = N-acetyl muramic acid

PBS = phosphate buffered saline

SPG = sucrose phosphate glutamate

DTT = Dithiothreitol

NOD = Nucleotide-binding oligomerization domain (NOD) proteins

SEDS = shape, elongation, division and sporulation proteins

PFA = Paraformaldehyde

DMSO = Dimethyl sulfoxide

RNA FISH = Fluorescent in situ hybridization targeting ribonucleic acid molecules

*meso*DAP = Meso-Diaminopimelic acid

qRT-PCR = quantitative reverse transcriptase polymerase chain reaction

Alr = alanine racemase

Ddl = D-alanine—D-alanine ligase

BME = β -mercaptoethanol

PBPs = Penicillin binding proteins

E. coli = *Escherichia coli*

B. subtilis = *Bacillus subtilis*

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Appendix 4

Activity and contributions 2015-2019

Year	Activity	Location
2015	Oxford Tropical Network conference	Phnom Penh, Cambodia
2015	Croucher's advanced microscopy course	Hong Kong
2016	Super resolution microscopy advanced training with Dr Graham Clark	A*Star, Singapore
2016	Microscopy course	Siriraj University, Bangkok, Thailand
2016	Rickettsia meeting, Mahidol Oxford Tropical Research Unit, presentation	Vientiane, Laos
2016	'The New Microbiology' EMBO course, poster presentation	Spetses, Greece
2016	Lab visit: Dr Michael Way (septins and microscopy)	Francis-Crick Institute, London, UK
2016	Lab visit: Dr Jost Enninga (live microscopy of the cytoskeleton)	Pasteur Institute, Paris, France
2016	Mahidol Oxford Tropical Research Unit student poster competition (winner)	Bangkok, Thailand
2017	Rickettsia meeting, Mahidol Oxford Tropical Research Unit	Bangkok, Thailand
2017	Lab visit: Dr Serge Mostowy (9 month project on the cytoskeleton of <i>Shigella flexneri</i>)	Imperial College, London, UK

2018	BSL2/BSL3 set up	Public Health Research Institute, Rutgers University New Jersey, USA
2018	American society of Rickettsiology conference, poster presentation	Wisconsin, USA
2019	American society of Rickettsiology conference, poster presentation	New Mexico, USA

Appendix 5

Publications 2015-2019

Improved Quantification, Propagation, Purification and Storage of the Obligate Intracellular Human Pathogen *Orientia tsutsugamushi*, Giengkam S, Blakes A, Utsahajit P, Chaemchuen S, **Atwal S**, Blacksell SD, Paris DH, Day NP, Salje J. 2015. *PLoS Negl Trop Dis*

Live imaging of the genetically intractable obligate intracellular bacteria *Orientia tsutsugamushi* using a panel of fluorescent dyes, **Atwal S**, Giengkam S, VanNieuwenhze M, Salje J. 2016. *J Microbiol Methods*

Evidence for a peptidoglycan-like structure in *Orientia tsutsugamushi*, **Atwal S**, Giengkam S, Chaemchuen S, Dorling J, Kosaisawe N, VanNieuwenhze M, Sampattavanich S, Schumann P, Salje J. 2017. *Mol. Microbiol*

Shigella MreB promotes polar IcsA positioning for actin tail formation, Krokowski, S, **Atwal S**, Lobato-Marquez, D, Chastanet, A, Carballido-Lopez, R, Salje, J, Mostowy, S. 2018. *J Cell Sci*